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ABSTRACT

This selected collection of How-To-Do-It articles published in the American Biology Teacher during the past six years presents experiments that can be conducted safely under properly trained and responsible teacher supervision. Contents include: (1) "General Biology and the Nature of Science"; (2) "Cells and Molecules"; (3) "Microbes and Fungi"; (4) "Genetics and Biotechnology"; (5) "Plants"; (6) "Animals"; and (7) "Ecology and Evolution". (YDS)

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Biology Labs That Work:
The Best of How-To-Do-Its, Volume II

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Biology Labs That Work:
The Best of How-To-Do-Its, Volume II

Edited by
Suzanne Black
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While every effort was made to anticipate questions and situations that could arise, the safe implementation of these activities must depend on the good judgment of teachers and is the responsibility of the local school district/institution. For this reason, NABT disclaims any legal liability for claims arising from use of these experiments. This information has been provided to teachers and to schools as a service to the profession; and NABT provides this material only on the basis that NABT has no liability with respect to its use.

NABT believes that under the guidance of a properly trained and responsible teacher, these experiments can be safely conducted in the classroom or laboratory. Before conducting these activities, NABT recommends that teachers check with their science supervisors, district or state education offices, and/or their local authorities for general lab safety and disposal guidelines. For further information on working safely with microorganisms, refer to NABT's publication, *Working with DNA & Bacteria in Precollege Science Classrooms*. Also, for more information on the safe handling and disposal of microorganisms and laboratory reagents, consult the current *Flinn Chemical Catalog/Reference Manual*, Flinn Scientific, Inc., Batavia, Illinois.

ABOUT THE COVER

Just like any good engineer, Kevin Loughran checks the seal on the construction of his Winogradski column before placing his mud mixture in the column. The funnel is made from the top portion of a 16-oz soda bottle to channel mud mixture into the bottle. Photo by Kathy Frame, NABT Director of Education

Preface

> *Biology has undergone colossal changes... The molecular revolution vastly complicates the task of balancing the curriculum. Somehow, we must convey the excitement of modern biology without omitting the fundamentals. As molecular discoveries crowd the stage, there is a strong tendency for less spectacular (but still vital) topics to slip quietly out of sight. The endangered topics include, for example, such matters as how the kidney works, how cells divide, how trees grow wood, and why we catch colds. Every young biologist used to learn such things. Today, it's a continual struggle to keep them in view. Solving that problem will be a major challenge in the 2000s.*[1]

To help teachers meet this challenge, we present *Biology Labs That Work: The Best of the How-To-Do-Its, Volume II*. We have tried to select the *best How-To-Do-It* articles published in *The American Biology Teacher* over the past six years. You will find laboratory investigations on exciting and vital topics. The first collection, *Biology Labs That Work: The Best of How-To-Do-Its*, was very popular. (Perhaps some of you, like us, no longer own a copy because you made the mistake of "loaning" it to a "forgetful" colleague!)

As in the first *Best of How-To-Do-Its* collection, the articles chosen for *Volume II* describe experiments and activities that are safe, dependable, feasible, economical and unique. You are sure to find a new activity among them—or, perhaps a fun, modern twist on an "old standby."

The list of authors of these articles reads like a *Who's Who* in biology teaching. You have probably attended their workshops, been taught by them, or collaborated with them. Without their desire to unselfishly share their work with the readers of *ABT*, this book would not exist. We also thank Chris Chantry, Kay Acevedo and Cheryl Merrill of NABT's Publication Department for giving us the opportunity to produce this book as well as the National Association of Biology Teachers as a whole.

Whether you're a new teacher focusing on building a resource collection, or a veteran teacher seeking new sources of inspiration, we hope you enjoy this book.

Suzanne Black
Randy Moore
Heidi Haugen

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[1]Thornton, R. "Thirty Years (1790–2000) in the Division of Biological Sciences." Interview. By Debra Cleveland. *News from the Division of Biological Sciences*. Winter, 2000: 2.

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General Biology & the Nature of Science

Using *Daphnia* To Teach Critical Thinking About Biological Research

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One of the classic laboratory activities for students at elementary, junior and senior high levels has been the treatment of water fleas (*Daphnia*) with various chemicals in order to show their effects on heart rate. The heart rates of the animals are taken before and after the treatment, and conclusions are drawn regarding the effects of the chemical. After years of attempting to get some consistent lab data from this activity, I have decided that it doesn't really teach students about the expected effects of stimulants, depressants and hormones on heart rate. Its real value is found in what it teaches about variations in the response of living things to chemicals and the difficulty researchers encounter when doing experiments with living things.

Background

Activities involving *Daphnia* heart rates can be found in a number of laboratory textbooks (see references). The suggested techniques are all very similar. Students are generally instructed to count heartbeats and record as beats per minute. They are then instructed to remove some or all of the liquid from around the animal and replace it with an experimental chemical solution. A few lab manuals instruct students to wait for a specific amount of time before taking the heart rates on the chemically treated animals.

The first year I tried this lab exercise, I found that many individual animals did not show the expected results of being treated with alcohol or nicotine. I responded by instructing students to pool their data. I was surprised to discover that even if data were collected from all biology sections, results were still too inconsistent to draw definite conclusions. For example, it was not unusual for nearly half the animals tested to show increased heart rates in alcohol while the rest showed decreases.

Next, I had students suggest ways to improve the experiment. These are the ideas that resulted from class discussions about the activity:

1. Mix all the chemical solutions in the same water in which the animals are cultured.
2. Make sure all the solutions are the same temperature as the culture.
3. Count heart rates multiple times during a period of 5–15 minutes after the chemical is added.
4. Try other chemicals such as caffeine, adrenalin and acetylcholine.
5. Do blind tests and double blind tests so that no one (including the teacher) knows what is being placed on the animals.
6. Attempt to keep the solutions at about the same concentration for each animal by removing all the water from the slide before putting the chemical solution on the animal.
7. Use some animals as a control group and remove the culture water and replace it with more water from the culture dish.
8. Design a better way to count the heartbeats or at least find a way to make the animals less active.

Over the years, classes have tried all of these suggestions, and lab results have shown little improvement. The data that made us the most skeptical were the control data. When water was drawn from the slide and replaced with water from the same culture container, heart rates in nearly 70% of the animals changed significantly.

Students have suggested, and I agree, that counting errors may be the biggest cause of inconsistency in results. Accurate counts are very difficult to make no matter what technique is used because the animals move around frequently and their heart rates are fast (180–220 beats per minute).

It is quite possible that the data for these experiments need to be studied using standard methods for statistical analysis. Because this lab activity has been designed for beginning science courses, such mathematical analysis has not been written into lab manuals. It would, however, be an excellent modification for advanced classes.

Conclusion

With lots of modifications, it appears that better results can sometimes be obtained. However, the most important change I made in the activity is the focus of the lesson. I now use it to teach about the problems involved in doing research with living organisms.

Every year my classes discuss the confusion that results from reading about research. This is especially true in the area of medical research. Articles appear almost weekly in newspapers and magazines that tell of experiments which support or conflict with previous research. Which substances really cause a significant risk for cancer? Can calcium supplements reduce the risk of osteoporosis? How much of the supplemental calcium and vitamins consumed are actually used by the body? Are there foods that can actually reduce cholesterol in the blood?

When my classes discuss news articles, I encourage them to read critically. Questions are raised about the content of the article. Who did the research? Who paid for it? How many subjects were tested? Were the tests performed on animals or people? Of those tested, what percentage displayed the results described in the article? How were the tests performed? Did the tests involve a blind or double blind situation? Could the results be repeated in other laboratories?

I find some irony in the fact that this time-honored laboratory exercise doesn't consistently give the results that are expected. Yet, if we want students to be doing science instead of memorizing facts and completing cookbook lab exercises, experiments with *Daphnia* can provide very effective learning experiences.

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A Little Leaven Leaveneth the Whole Lump

Using *S. cerevisiae* To Investigate Life in a Biology Classroom

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"If a jack-rabbit is hopping along I-80, and is suddenly mowed over by a semi-truck, what's the difference, in that split second, between the two rabbits—living and dead?" My high school AP Biology teacher[1] allowed us all to pause and think—same cells, same organelles, same enzymes, same DNA—what's the difference? As we tried out different answers to the question, we began a debate that continued throughout the year. The question still remains with me. The difference between living and nonliving is the central point of Biology, the Study of Life. As I study biology with my own students now, I try to pose questions to students and to prod them to pose their own questions that make them want to search for answers.

I want that puzzling notion of "life" to be the focus of our investigations. That means we have to start by looking at living organisms; frogs in formaldehyde demonstrate many useful principles, however life is not one of them. Constraints—time and money, primarily—limit the choices that are appropriate, and yet that is not a problem. The simpler the organism, the easier it is to demonstrate that the principles governing life apply to all organisms. It is only a small stretch for the students to extrapolate these ideas to their own favorite organisms, themselves. I have found that *Saccharomyces cerevisiae*, yeast cells, provide an excellent system for student-designed experiments.

Doing a Lab

Often, high school biology "labs" are as solid and unresponsive as the tables, chairs and glassware in that laboratory. A "lab" is not something that you *do*, it is a *place* where you do something: an experiment. Doing an experiment requires posing a question. If a student only follows the steps in the protocol a teacher hands out, then no question has been asked, and no answer is forthcoming. She can only hope she does well enough that her "answer" will conform to those of all her classmates. In a ninth grade introductory biology class, I have encouraged students to *design experiments*, rather than *perform labs*. I began with several criteria to focus the experiments:

1. The students should understand a system. In a research laboratory, scientists do not skip back and forth from *Drosophila* to *Caenorhabditis elegans* to *Escherichia coli*. They use one system, come to understand it, and then pose questions from within that system.
2. Each experiment should build on past work. A single experiment is rarely enough to tell the full story. All observations need to be verified by experiments that test other approaches to the same problem.
3. The students must design their own protocols. The teacher can provide tools that will aid the process, but the overall designs of the experiments must be their own.
4. Experiments must be allowed to "fail." A poorly designed experiment will not yield usable data, nor will a well-designed experiment that poses the "wrong" question, or uses the "wrong" techniques.

Trypan Blue

I picked yeast as our system, and began the year by giving the students something to wonder about. Each lab group received two slides of yeast cells stained with trypan blue and was asked to compare them, note any differences, and then share their results with the classroom. The students found many differences, and described the cells on one or the other of the slides as more clumped, puffier, more "mobile," or smaller. When we compared the groups' results on the board, we found that every group had one characteristic in common: there were more blue cells on one slide than on the other. Puffiness, size, and all other observations now looked artifactual; one slide had blue cells, one slide had white cells (Figure 1). The next question had to come from the students: Why blue cells?

Every group formed a different hypothesis and set out to test it. Controlled experiments tested whether freezing and thawing, longer staining, growth time, type of food, or temperature would explain the difference. Only one group found a significant difference between their samples: the population of cells heated to 60° C for 10 minutes increased from 20% blue to more than 90% blue.

Heat

Now the students had some usable information and were ready to ask another question: Why heat? This, however, is not a simple question that can be answered with one experiment. An argument began in the back of the room—"If you put the dye in while the cells are still hot, of course they will absorb more dye! You haven't changed the cells at all." As a result, the students proposed a second experiment, and in their debates determined that they should try three variations. One group would heat the cells exactly as before, but would vary the time the cells were exposed to heat, from 0 to 30 minutes. Another group would test temperature as the variable, with samples incubated at temperatures ranging from freezing to boiling. The third would heat the cells to the same range of temperatures, but would allow the cells to cool for 30 minutes before staining them.

Most readers have probably guessed the results already: the cells were predominately white to a temperature of 40° C. After that point, more stained blue as the temperature increased, showing a strong correlation between temperature and percent of blue cells. Similarly, the longer the cells remained

[1] G. Merrill Webb, recognized by NABT as one of the nation's Outstanding Biology Teachers in 1998.

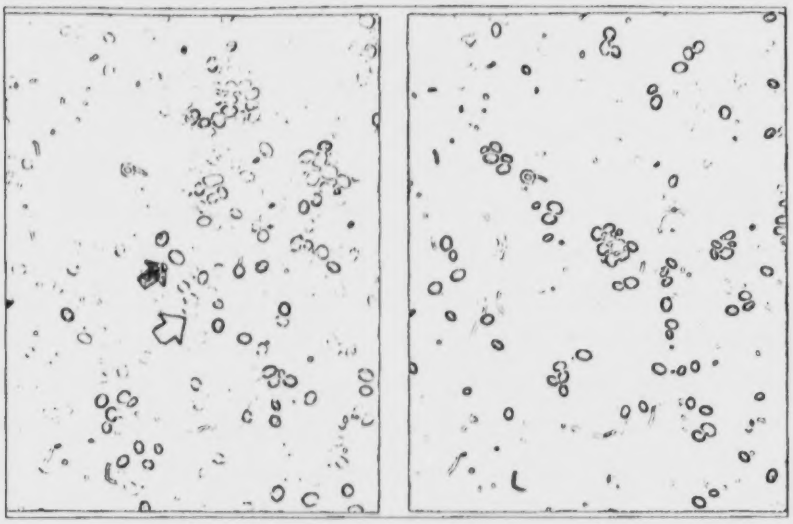

Figure 1. *Heat-treated cells stained with trypan.* Photo on left shows untreated cells that were stained with an equal volume of 0.4% trypan blue, buffered with 0.81% sodium chloride and 0.06% potassium phosphate, dibasic. Closed arrow indicates a positively staining cell, while open arrow indicates a cell that resists the stain. Photo on right shows cells that have been boiled for 5 minutes and stained as above. All of the cells are stained with trypan blue.

at 60° C, the greater the number of cells stained blue. Cooling the samples before staining them did not make any difference.

Viewing the data, one student concluded: "There must be some permanent chemical change that heat brings on to make them turn blue." I posited that the chemical change could be something we call death. They argued at first, perhaps because too many TV shows had led them to expect something more dramatic from death than what they had seen. Now they have had a semester to challenge the idea. During that time they have come to learn the differences between living and nonliving—not a simple question, even for a yeast cell.

Expanding

Each unit that we study relates to the yeast system well. While studying membrane transport, I explained how trypan blue is excluded from cells by active transport. The students made the logical jump that heat must, therefore, affect the ability of the transport protein to function. Protein denaturation and its consequences are concepts that are now clear in their minds.

From that point, the students themselves have taken the experiments in many directions. They have found that most structural features of yeast cells remain visibly unchanged despite the heat treatment. The cell membrane is still apparent, and dead yeast cells can still be made to shrink or swell in response to osmotic pressure. During respiration studies, they saw that heat-treated yeast cells did not produce the bubbles of CO_2 that can fill a balloon. A modification of Joseph Priestley's experiment showed that a beaker of yeast in a tightly capped jar lived longer (resisting trypan blue staining) if a green plant was placed in the same jar. The students used a respirometer to quantify oxygen consumption (The College Board 1997). Cell division occurred in living, but not dead, yeast, as attested by counts of budding yeast, bud scars, or density growth curves in a spectrophotometer. Again and again, the students demonstrated that the characteristics expected of a living organism can be observed in the white, stain-resisting cells, but not in the heated, blue-staining cells.

Questions

We have also had some surprises. Again and again, different groups found that oxygen consumption stopped completely at temperatures that only led to a slight increase in the number of blue-staining cells. "Human error," the students all wrote in their notebooks, having no confidence in their own findings, until they discovered the same result repeatedly. Are the membrane transport proteins in dead cells using their remaining ATP to remove the trypan blue, or have the living cells temporarily shut down cellular respiration, in a sort of torpor brought on by adverse circumstances? Some groups have decided to follow up on these questions by doing extra experiments during lunch and after school. I have limited my role to helping them refine experimental designs or steering them toward papers on heat shock proteins.

Refining & Repeating

The students' lack of expertise at the bench has often affected the results they obtained: they dropped test tubes and mixed up samples, but when the bell rang they still had to move on to geometry. Usually we salvaged some data. Only one experiment had to be discarded completely. The first time we set up the respirometers, yeast spilled out of the tubes and mixed with the KOH-soaked cotton balls. The volume of oxygen remained stubbornly constant for 45 minutes. We spent the next class period reviewing the experiment together. The students devised a better way to place the yeast samples in the vial, and we all crossed our fingers that, when we repeated the experiment, the ever-varying classroom temperature would exceed the 15° C we had shivered through the first time.

Never did the data from an experiment produce the perfect arcs and lines on a graph that students see in a textbook and come to expect. Inevitably, one data point would shoot drunkenly off a perfectly good line, yet many of the lines became considerably smoother when additional data from other groups' work were added.

Conclusion

As much as I was excited about the idea of student-designed experiments, I feared putting the course into their hands. What if they didn't come up with any good ideas? The group of 20 14-year-olds devised a variety that far exceeded my own. Sometimes I demonstrated a tool, like the respirometer, and then allowed them to decide how they wanted to use it. As the year progressed, they developed more and more of their own tools, and they applied them in new situations to new questions. Lab days have been hectic; almost no two groups are asking the same question or running the same experiment. Wrap-up days, when results are shared, have been enlightening.

At the end of the first semester, each student produced a paper titled "Trypan blue is a vital stain for yeast cells." Like the papers in many scientific journals, the title is a sentence with a verb. The Materials and Methods section is long; they have accumulated evidence from many independent experiments to demonstrate the point. The Results section includes many separate figures, each with a graph of the results from a different experiment. No two papers have the same set of data. True, the results are not surprising to anyone who ordered the trypan blue from the "vital stains" section of a chemical supply catalog, but the students have independently supported that description with their own data.

This group has begun to tire of yeast cells ("If I see ONE more blue cell . . ."). It is a good time for break-off groups to start some work on their own, while the rest of the class moves on to a new system. The experiments with yeast, however, could go on and on. Mutant strains with unusual nutritional requirements would demonstrate the need for a source of carbon. Those same strains could be used later in studies of Mendelian genetics and natural selection, and in molecular biology experiments with genetic transformation. Meiosis and sexual reproduction could be demonstrated with MATa and α strains (Johnston 1994).

S. cerevisiae, cheap and available in the dairy section of the local grocery store, provides an excellent system for students to enter inquiry-based science. As biology teachers, we are all trying to encourage our students to wonder and to question. The foundation of the classroom is the question, and the question, "What is 'living'?" is the foundation of the study of biology.

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Revisiting Antony van Leeuwenhoek

Using a Student-Constructed Simple Microscope To Stimulate Interest in Microscopy

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. . . I am writing to tell you that a certain most ingenious person here, named Leeuwenhoek, has devised microscopes which far surpass those which we have hitherto seen . . . (He) . . . describes certain things which he has observed more accurately than previous authors . . . if it pleases you, and you would test the skill of this most diligent man . . . give him encouragement, then pray send him a letter containing your suggestions, and proposing to him more difficult problems of the same kind.

—Reinier de Graaf (28 April 1673)[1] (Gravé 1991)

In 1673, Antony van Leeuwenhoek, a Dutch draper and haberdasher with little or no formal scientific training, wrote a most extraordinary letter to the Royal Society of London. In fascinating detail, van Leeuwenhoek described the ". . . observations on a mold; on the stinger, mouthparts, and eyes of the bee; and on the louse . . ." he made using the simple microscopes he designed and crafted by hand (Gravé 1991). In the 109 additional letters to the Society that followed over the next half century, van Leeuwenhoek reported ". . . numerous wonders by the aid of the microscope. . . .," ranging from the first descriptions of bacteria and protista ("zeer kleine dierkens" or little "animalcules") to the circulation of blood in the capillaries of an eel (Stein 1931). In 1679, to honor "His work . . . in little things, but not little in glory," van Leeuwenhoek was elected a member of the Royal Society, a distinction rarely awarded to a person who is not a citizen of Great Britain (Stein 1931).

Today, many biology teachers begin their laboratory instruction on the compound optical microscope with a discussion of van Leeuwenhoek's life, the simple microscopes he crafted, the techniques he invented, and the microscopic world that so fascinated him. Some instructors have even constructed or purchased replicas of a van Leeuwenhoek microscope to demonstrate how van Leeuwenhoek used them (Walter & Via 1968).

This paper describes how we expanded a traditional discussion of van Leeuwenhoek's accomplishments into a hands-on laboratory activity in which each student builds his or her own van Leeuwenhoek-like microscope and uses it to study some of the ". . . most aesthetically pleasing images science can offer . . ." (Ford 1985). Once familiar with visual sense enhancement by use of the microscope, and with some experience observing the "microscopic world," we find these students more receptive, and often eager, to develop the techniques needed to use a modern compound optical microscope.

Construction of van Leeuwenhoek-like Microscope

As van Leeuwenhoek was aware, the most important component of a simple microscope is the lens; and he apparently devoted much time perfecting his lens grinding skills. However, lens grinding is well beyond the range of activities described in this laboratory experience. Fortunately, this is not an unsurmountable problem. Many craft stores and plastic supply houses sell inexpensive plastic "cabochons" or decorative "Round See-Thru Stones" which are actually excellent convex lenses ideally suited for our purpose.

Given simple instructions, either verbally or in writing, and supplied with the following readily available materials, students should be able to complete construction of a van Leeuwenhoek-like microscope (Figure 1) in about 45 minutes:

1. Photocopy of microscope "body" template
2. 15 × 15-cm piece of cardboard
3. Scissors
4. Rubber cement
5. 11-mm plastic cabochon (or "Round See-Thru Stone")
6. 1-inch "corner brace"
7. Premixed tube of wallpaper paste or other water-based adhesive
8. 6/32 × 1-inch round head machine screw and nut
9. #10 brass washer
10. Two 8-cm lengths of heavy duty self-adhesive waterproof foam insulation tape (½-inch thick and ¾-inch wide)
11. Two double pointed ½-inch staples
12. Plasticene "clay"

To begin the microscope assembly process, each student should cut out and then glue the microscope body template (Figure 2) to the cardboard backing using rubber cement. When the cement is dry, the student can cut out the microscope "body" template with its cardboard support. The two lengths of self-adhesive foam insulation are then secured onto the microscope body-cardboard support over areas marked "A." These foam strips will serve as the microscope "stage" and as a guide when focusing the optical system. To construct the optical system of the microscope, the student should apply a very small quantity of wallpaper paste or adhesive around one of the two screw holes in the corner brace and use this adhesive to attach the cabochon (flat surface toward the screw hole) over the hole. If excess paste gets onto the center (viewing portion) of the lens, the lens can be easily removed from the brace, washed with water, dried, and then reglued. The corner-brace optical system is secured to the microscope body using the machine screw, washer and nut as shown in Figure 1. The metric ruler strip that will be used to calculate the magnification of the microscope is cut from the photocopy and glued to one of the two foam insulation strips again using rubber cement. Finally, to complete the assembly of the microscope, two staples that will serve as microscope "stage clips" are inserted into the foam insulation strips as shown.

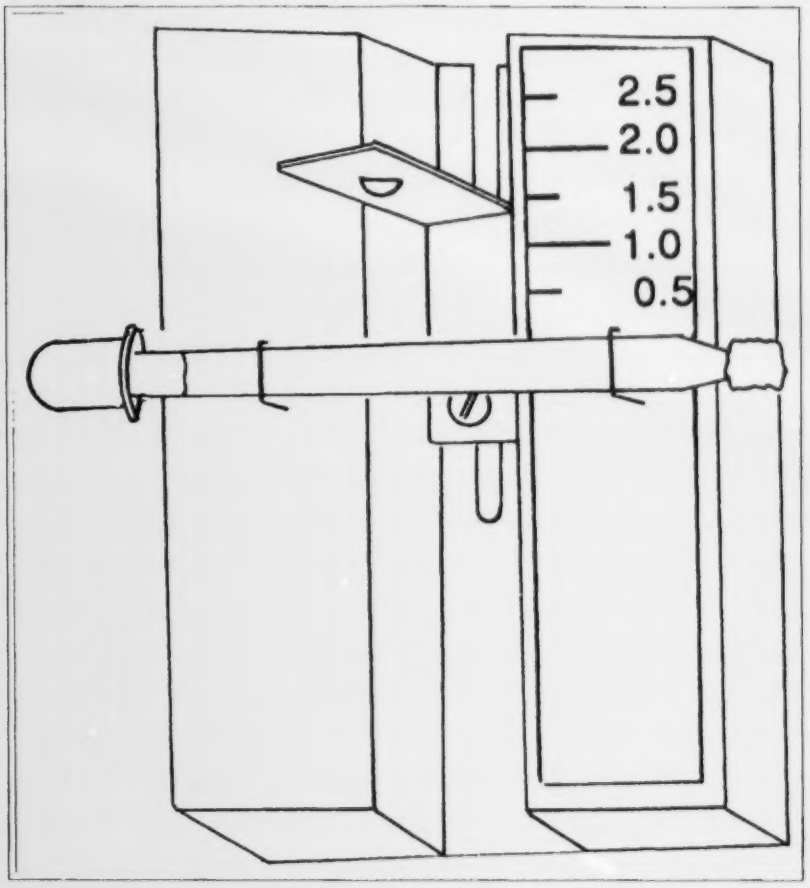

Figure 1. Completed van Leeuwenhoek microscope (not to scale).

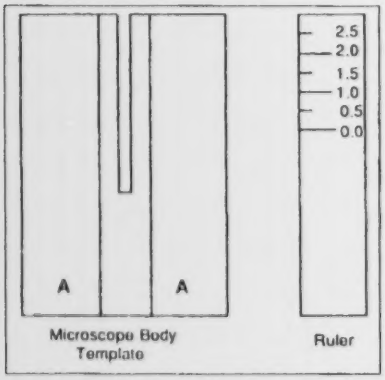

Figure 2. Microscope and ruler templates that can be photocopied and used by students to construct their microscopes.

Observation of Vinegar Eels

It is most appropriate that the student first turn his or her newly constructed van Leeuwenhoek-like microscope to an observation of the same vinegar eels (*Anguillula aceti*) that van Leeuwenhoek so vividly described in his letter to the Royal Society in 1676 (Dobell 1932). The student can fill a common eyedropper with liquid from an apple cider vinegar culture of vinegar eels (Morholt, Brandwein & Joseph 1958), seal the open end with plasticene "clay," and then secure the dropper onto the insulation tape "microscope stage" by sliding it under the two "stage clip" staples. Holding the microscope close to one eye, and pointing it toward a distant source of light (if skylight is used, caution the student not to point the microscope toward the sun!), the student can use the screw stem extending from the bottom of the microscope to move the brace-mounted lens back and forth until the eels are clearly in focus. With adequate illumination and proper focusing, the student should have very little trouble observing the rather large corkscrew-shaped eels swimming rapidly through the culture medium.

Figure 3. The completed microscope is held close to the eye using one hand and focused by sliding the screw stem back and forth using the other hand.

Once the student has made and recorded his or her observations, it can be most informative to estimate the magnification of the microscope system used to observe the vinegar eels. This can be accomplished using the metric ruler attached to the microscope. The student can determine the distance from the front of the convex lens to the middle of the eye dropper when the eels are in focus and use this distance measurement to calculate the magnification using the formula:

Magnification of the microscope
= 25 cm/distance from the lens to specimen when the eels are clearly in focus

Depending on the diameter of the cabochon used, magnifications between 25X and 45X are obtainable.

Familiar with the use of his or her microscope, the student can now be encouraged to embark on his or her own microscopic investigations using cultures of other large microorganisms made available to them. We have found that *Paramecium, Vorticella* and rotifers make fascinating subjects for follow-up microscopic studies. In addition, if two slits are cut in the foam insulation, temporary wet mounts and permanent slide preparations can be mounted and observed. We have also used this activity to introduce a study of cell structure. Students prepare

temporary mounts of slivers of cork and then compare their observations with Robert Hooke's classic description of cork "cells" in his *Micrographia*, published in 1665.

Several lines of evidence strongly suggest that students are indeed still as "... interested in viewing the very small as was Antony van Leeuwenhoek (1632–1723) more than three centuries ago" (Walter & Via 1968). Even though students are told that they need not take their microscopes with them when they leave class, few are ever found left behind. Moreover, many students come to the next class meeting with descriptions of observations they have made on their own, using the microscopes they built in class. There have even been students who returned to class proudly displaying more substantial and modified instruments they constructed from wood, plastic, and even sheet metal. Finally, there is general agreement among instructors that students who have participated in this introductory laboratory activity perform at a higher level of achievement in subsequent laboratory activities where the compound optical microscope is used.

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Chemotaxis in the Plasmodial Slime Mold, *Physarum polycephalum*

An Experimental System for Student Exploration & Investigation

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An important objective of laboratory instruction, particularly at the introductory level, is to provide students with opportunities to ask testable questions, design experiments to address these questions, implement their experimental designs, and analyze and present the results of their work. The challenge to realizing this objective is that it is often difficult to identify interesting and suitable organisms and experimental systems for undergraduates to explore in the laboratory. There are several criteria we have adopted for our selection of organisms used for experimentation by first year students. First, the organisms should be inexpensive and easy to grow and maintain. Second, the skills needed to manipulate the organism should be relatively straightforward so that students quickly gain competence to design their experiments. Third, student concern and sensitivity regarding animal use must be considered. Finally, the biology of the organism should suggest some obvious questions suitable for experimentation. With these criteria in mind, we have designed, for our introductory biology course, a laboratory unit in which students investigate chemotaxis in the plasmodial slime mold, *Physarum polycephalum*. This unit is designed so that students pose their own questions and perform experiments to answer their questions. Since analysis of experimental results always generates more questions, we provide students with the opportunity to set up another experiment to pursue an additional avenue of inquiry. Furthermore, the design of this laboratory unit allows us to teach about quantitation and simple statistics since all experiments are constructed so that data can be analyzed using the chi-square test.

Background

Physarum polycephalum, a plasmodial slime mold (myxomycete), lives in dark, moist environments such as under the bark of decaying logs and beneath decaying leaves on the forest floor. The vegetative form of this organism is the plasmodium, a glistening yellow mass of indefinite morphology. Plasmodia are negatively phototactic, display chemotaxis, and have the ability to "crawl" to seek food. Feeding on bacteria, spores and decaying organic material, a plasmodium can grow to a fairly large size (up to 30 cm in diameter). Despite this large mass, it is not composed of separate cells but is one huge amoeba-like cell containing many nuclei (Sauer 1982).

When food is scarce, the starving plasmodium undergoes a remarkable transformation. The plasmodial mass develops numerous fruiting bodies, called sporangia, which serve as a resting stage. When food becomes available once more, the spores of the fruiting body germinate and release either amoeboid or flagellated swarm cells depending upon environmental conditions. It is the swarm cells which fuse together to ultimately produce another feeding plasmodium (Cummins & Rusch 1968; Sauer 1982).

For more than 35 years, *Physarum polycephalum* has been a model research organism for the study of several important biological problems including growth and differentiation, cell cycle dynamics, cytoplasmic streaming, and cytoskeletal function (Cummins & Rusch 1968; Sauer 1982). Because it is easy to grow and maintain, *Physarum polycephalum* has also been used for laboratory instruction as well (Holmes & Stewart 1977). We report here the design and results of an introductory biology laboratory unit which focused on the chemotactic behavior of plasmodia.

Protocol

Overview

The chemotaxis laboratory unit has several specific objectives. During the first week, we provide plasmodia and several materials for students to test for chemotactic response by plasmodia. Initially, all students examine the general morphology of plasmodia and observe cytoplasmic streaming. Next, each lab group designs and implements an experiment to test the chemotactic response of plasmodium to one specific substance. Students collect their results and hand them into their instructors so that data can be pooled for the entire class (five lab sections) and made available for the students during the second laboratory meeting. Although we wait until all experiments are complete before discussing and using the chi-square test, we do talk about experimental design, probability, and the concept of the null hypothesis during this first meeting. During the second week, results from all experiments are made available. Each lab group sets up another chemotaxis experiment, the designs of which are based on the first sets of results. The data collected from this second set of experiments are also pooled. Finally, during the third week, data are analyzed using the chi-square test.

Student Instructions

Week #1

A. Initial Observations

1. Without removing the cover of the petri dish, examine the stock plate of *Physarum*. The plasmodium is the yellow, glistening, web-like material on the dish.
2. Use a dissecting scope to examine the flow of cytoplasm in the plasmodium. Is the flow directional? Does the direction of flow change?
3. What is the probable function of the cytoplasmic streaming?

B. Chemotaxis in *Physarum*

Physarum is repelled by or attracted to some substances, and does not

respond to others. In today's lab, your group will test whether *Physarum* exhibits a chemotactic response to one of several available test substances:

1. 100 mM glucose in 1.5% agar
2. 20 mM NaCl or KCl in 1.5% agar
3. Sweetened cereal (several types are available)
4. Unsweetened oatmeal
5. Fresh fruit

State the null hypothesis for your experiment. After discussion with your lab partners, predict how *Physarum* might respond to your test substance. Try to formulate an explanation for your prediction.

Experimental Procedure

There is a variety of ways to set up a chemotaxis test for *Physarum* but to make this experiment suitable for chi-square analysis, the plasmodium should be presented with two choices for directed migration. Sterile technique will be used to set up this experiment. Your instructor will review the necessary procedures with you.

a. Each group needs a plasmodium culture, five agar plates (1.5–2% agar), a test substance, and a scalpel.
b. Cut blocks of agar from one non-nutrient agar plate and from the glucose-agar plate; these blocks should be approximately 1 cm^2.
c. On each of four non-nutrient agar plates, deposit one non-nutrient agar block approximately 1 cm from the edge of the dish. If your test substance is dissolved in agar, on the opposite side of one of these plates, deposit a second agar block, also approximately 1 cm from the edge of the dish. If your test substance is not in agar, sprinkle or place a small amount of the food item on the opposite side of the plate. Be sure to mark the bottom of the petri dishes to indicate the identity of each type of agar block or food item. (See Figure 1).
d. Cut the plasmodium culture into 1-cm^2 blocks. Transfer an agar block containing a piece of plasmodium to each of the three petri dishes. When choosing areas of *Physarum* for transfer, try to select a thick "vein" located towards the edge of the plasmodium. Place the agar block, *plasmodium* side down, in the center of the dish. Be careful not to transfer pieces of oatmeal from the stock cultures.
e. Draw a line down the middle of the bottom of the petri dish to demarcate the left and right sides and label left/right.
f. Wrap the dishes in aluminum foil and incubate right side up at room temperature.
g. Observe plasmodium migration at ~20–24 hours and record its location. A plasmodium positioned anywhere besides the center can be scored as a + for that half of the dish.
h. We will pool class data. Your instructor will discuss with you how to record your results on the data sheets.

Week #2

Followup Studies

The results from week one will be used to determine what you will do for your second set of experiments. Some possible projects include:

1. Determine the rate of response to a test substance. Transfer an agar block containing a test substance onto a non-nutrient agar plate. On the other side of the plate place a non-nutrient agar block. After 24 hours, place an agar block with *Physarum* in the center of the plate. Arrange with your lab partners to observe the plates at two-hour intervals.
2. Test for food preference. If *Physarum* is attracted to more than one test substance, set up an experiment in which it is provided a choice between two of them.
3. Test the response of *Physarum* to a combination of an attractant and a repellent.
4. Test an idea of your own after approval by your instructor.

Week # 3

Chi-Square Analysis of Data

For this experiment, the null hypothesis is that plasmodia are *not* migrating directionally; migration is random. Since a minimum sample size of 15 is needed for calculating the chi-square test, you will use pooled results for analysis. We will discuss how to do the chi-square test and analyze results together in lab.

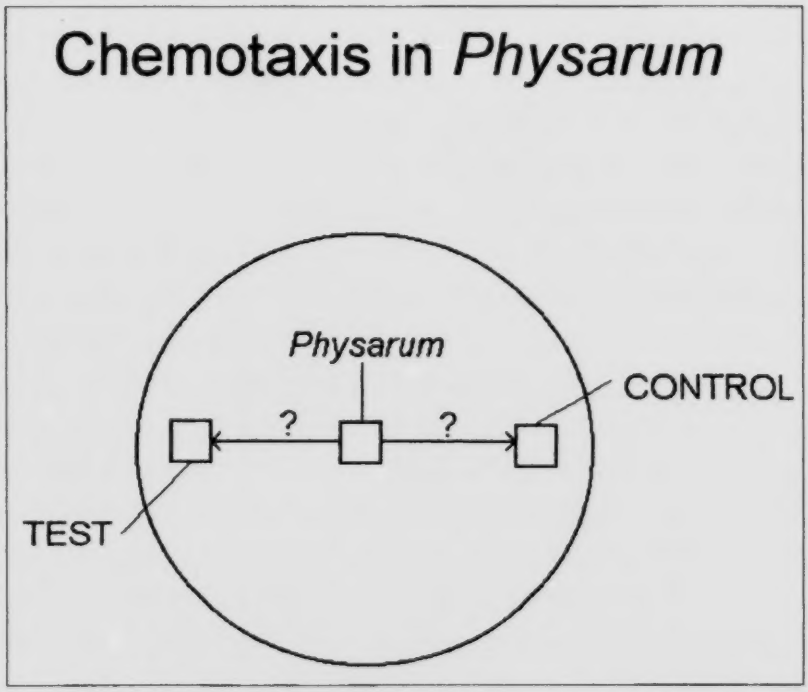

Figure 1. Experimental setup for chemotaxis assay. The control is a block of 1.5–2% agar. The test block is either agar containing a specific test substance or it can be a small pile of food or some type of solid material. The *Physarum* block consists of a piece of agar with a piece of plasmodium on its surface.

Laboratory Preparation Instructions

Growing *Physarum*

Preparing cultures of *Physarum* plasmodia is quite straightforward. You can obtain cultures from Carolina Biological (2700 York Road, Burlington, NC 27215-3398) or other supply companies. Order either the plasmodium or preferably, sclerotium stage, which is a resting structure (Carolina Biological Catalog #15-6190). Cultures are grown on sterile 1.5–2% agar (15–20 g agar per liter of distilled or deionized H_2O) with approximately 25–30 oatmeal flakes sprinkled on top after the agar has solidified. Using a sterile scalpel, cut a block of agar on which a piece of plasmodium is present, and transfer it to the agar-oatmeal plate with the plasmodial side facing down. If culturing a sclerotium, use sterile forceps to transfer a piece of filter paper containing the resting stage (this is how they arrive from Carolina Biological) to the agar-oatmeal plate, with the sclerotium facing down. Wet the filter paper with a drop of sterile H_2O.

Once cultures are set up, seal the edges of each plate with parafilm or plastic, electrical tape, and wrap dishes in aluminum foil to keep out light. Cultures started from plasmodia probably need to be transferred every three to four days if kept at room temperature. Cultures initiated from sclerotia may need one to two days of incubation before growth commences. However, plasmodia will go into "suspended animation" for weeks (probably months) if refrigerated.

Chemotaxis Tests

As discussed in the Student Instructions, plasmodia can be presented with blocks of agar containing test chemicals or pieces of solid food. In addition, the assay will also work if sterile pieces of filter paper (~1 cm^2) soaked in the test chemical are used instead of agar blocks. The advantages of the agar blocks are that the test chemical won't drip out of them and it is easier to deliver comparable amounts of test chemicals for all replicates.

Chi-Square Test

To ensure that students gain a firm understanding of chi-square, we discuss this topic and do simple analyses as a group in the laboratory. We supplement this discussion with a reading assignment; information about chi-square analysis can be found in most general biology or genetics lab manuals (for example, see Eberhard 1990).

Scheduling

Although this unit takes place over a three-week period, it does not require three complete (three-hour) laboratory periods. The work entailed for Weeks 1, 2, and 3 can be completed in 1–1½, ½–¾, and 1–1½ hours, respectively.

Student Results

During the first week of the lab unit, students tested for the chemotactic response of *Physarum* plasmodia to five different substances. A positive chemotactic response was observed when plasmodia were presented with glucose, banana, Corn Flakes,™ or Sugar Pops,™ but not NaCl (Table 1). These results generated new questions that students tested in the next series of experiments. After observing the positive response of plasmodia toward both glucose and Sugar Pops, several groups elected to test the responses to sweet versus sour candy (Gummi Bears™ and Sour Patch Kids,™ respectively). Their results showed a marginal positive response for the sweet candy and a strong negative response to sour (Table 2). The observation that plasmodia were strongly attracted to sweetened cereal, Sugar Pops (Table 1), led to more experiments. Several groups presented plasmodia with the choices between Sugar Pops and plain agar, sucrose and plain agar, and Sugar Pops and sucrose. Other groups compared the responses of plasmodia to cornflakes versus Sugar Pops (a corn cereal). Results from these two experiments showed that while they are somewhat attracted to sucrose, some other component in the Sugar Pops and especially the Corn Flakes is even more attractive (Table 2). Finally, some groups considered the attraction of plasmodia to glucose and the lack thereof to NaCl. They designed an experiment to examine whether plasmodia would cross a salt barrier to get to glucose. When presented with the choice between glucose surrounded by NaCl or plain agar, plasmodia showed no preference. In contrast, the choice between glucose surrounded by NaCl versus plain agar surrounded by NaCl showed that plasmodia would indeed cross a salt barrier to reach glucose (Table 2). Apparently, if salt concentrations are similar on both sides of the plate, the plasmodia respond to glucose.

Outcomes

Students enjoyed this unit very much and approached their work with enthusiasm. Because students were presented with an experimental system and then invited to ask their own questions, they felt very much in charge of their work. As a consequence, they learned quite a lot about experimental design, data analysis, and simple statistics. Moreover, they experienced one of the most important aspects of science, namely the continuous generation of questions from experiments done to answer other questions. In fact, the second set of experiments generated another round of questions, as well as some very good ideas for improvements in experimental design. Some of the ideas include testing varying concentrations of different sugars, vitamins, amino acids, and salts; examining whether plasmodia are sensitive to pH (and whether the attraction to specific test chemicals will be influenced by pH); trying to extract the attractant present in various cereals and the repellent in Sour Patch Kids; and exploring the relative responses of plasmodia to light and chemoattractants.

Acknowledgments

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Table 1. Chemotactic responses of *Physarum* plasmodia: Class results from experiments run during Week #1.

Test Substance	*Movement Towards Test Substance*	*Movement Not Towards Test Substance*	*Chi²*	*p Value*
Glucose (100 mM)	19	4	9.78	$p < .01$*
NaCl (20 mM)	12	8	0.80	$p > .3$
Banana	19	1	16.20	$p < .001$*
Cornflakes	12	2	7.14	$p < .01$*
Sugar Pops	11	1	8.33	$p < .01$*

* Statistically significant.

Table 2. Chemotactic responses of *Physarum* plasmodia: Class results from experiments run during Week #2.

Experiment A: "Sweet" vs. "Sour"			
Movement Towards Sweet	Movement Towards Plain Agar	Chi^2	*p* Value
10	3	3.13	$p > .05$
Movement Towards Sour	Movement Towards Plain Agar	Chi^2	*p* Value
0	9	9.0	$p < .01$*
Movement Towards Sweet	Movement Towards Sour	Chi^2	*p* Value
11	0	11.0	$p < .001$*
Experiment B: Sweetened Cereal vs. Sucrose			
Movement Towards Sugar Pops	Movement Not Towards Sugar Pops	Chi^2	*p* Value
15	1	12.25	$p < .001$*
Movement Towards Sucrose	Movement Not Towards Sucrose	Chi^2	*p* Value
10	4	2.57	$p > .01$*
Movement Towards Sugar Pops	Movement Towards Sucrose	Chi^2	*p* Value
12	2	7.14	$p < .01$*
Experiment C: Corn Flakes vs. Sugar Pops			
Movement Towards Cornflakes	Movement Towards Sugar Pops	Chi^2	*p* Value
15	5	5.0	$p < .05$*
Experiment D: Will Physarum *Cross a Salt Barrier To Reach Glucose?*			
Movement Towards Glucose Agar Surrounded by 20 mM NaCl	Movement Towards Plain Agar	Chi^2	*p* Value
8	5	0.82	$p > .3$
Movement Towards Glucose Agar Surrounded by 20 mM NaCl	Movement Towards Plain Agar Surrounded by NaCl	Chi^2	*p* Value
13	2	8.07	$p < .01$*

*Statistically significant.

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The Red & White Yeast Lab

An Introduction to Science as a Process

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This article describes an experimental system based on an engineered strain of bakers' yeast that is designed to involve students in the process by which scientific knowledge is generated. The yeast grow to form a reproducible pattern of red and white; the students' task is to find out why. To solve this problem, students generate their own hypotheses, design their own experiments, and interpret their own results. Although the experimental techniques are simple, the puzzle is challenging. The richness of this exercise does not lie in the answer itself, but in the discussions among the students as they try to find the answer.

The Experimental System

The goal of this exercise is to allow students to experience the interaction between hypothesis and experiment. It is designed to show students how we know what we know—that the material in the text didn't come carved in stone, but was the result of a complex process that they could participate in and understand.

In order for this process to be comprehensible to students with limited biology background, the phenomenon being investigated must be simple to grasp, and yet have several possible explanations. I devised an exercise based on an engineered (Koshland et al. 1985) strain of bakers' yeast (*Saccharomyces cerevisiae*). Others have used related strains in other open-ended teaching lab experiments (Manney 1997a).

Like other microbes, yeast can be grown on solid nutrient medium; at room temperature, in about a week, a single cell divides to produce a visible colony. When a large patch of the engineered strain is grown on solid medium, a deceptively simple pattern appears: The center of the patch is red and the outside edge is white (see Figure 1; color pictures are available on-line [White 1999]). The students' task is to find out what they can about why the center is red and the edge white.

Figure 1. The phenomenon: a patch of yeast growing on a plate. Why is the center red and the edge white?

The Exercise

The exercise takes three 90-minute lab sessions, each separated by a week. The teacher begins the first session by showing students plates with the red and white patch and explaining the problem they must solve: "Find out what you can about why the center is red and the edge is white." The students are told that a satisfactory outcome would be to find some part of the answer or even to rule out some possible answers, and that the process is what is important.

Although very little specialized knowledge is required, a brief introduction to yeast biology is helpful to bring members of the class to the same level. Yeasts are unicellular microorganisms that reproduce by budding. Budding takes place roughly every 90 minutes under optimal conditions. This leads to rapid growth at the beginning, although the cells soon begin to grow more slowly as they deplete the nutrients in the local area of the plate. The budded cell is usually, but not necessarily, identical to the mother cell.

The class then breaks up into groups of three to discuss possible explanations for the phenomenon and questions they'd like to answer experimentally. Table 1 summarizes some typical questions and hypotheses. Each group then presents a model or a question that is written on the board. At this point, it is important not to edit what the students say or try to guide them; consistency with their experimental results will be the criterion for evaluation during the second session.

The students are then given the tools: fresh plates and sterile toothpicks (Figure 2). They also receive a spare plate with a patch and some nonsterile toothpicks so that they can acquire a feel for the material as they design the experiments they will perform. Each group then describes to the class the experiments they will perform and how their experiments will address the problem at hand. They are also encouraged to try experiments from other groups.

Before they begin their experiments, it is useful to go over proper sterile technique. Contamination is a serious problem, since the medium used is very rich and the plates must grow

Table 1. Some initial questions & hypotheses.

Questions

1. Is white stuff yeast? A waste product? Live yeast? Dead yeast?
2. Is red dead? Dormant? A waste product of white?

Hypotheses

1. White protects red.
2. Cells started red and turned white.
3. Two kinds of yeast, red and white and white grows faster.
4. There is ***one strain*** and color depends on:
   (a) Crowding (crowded cells are red).
   (b) Waste accumulation (cells exposed to high waste levels become red).
   (c) Nutrient level (cells exposed to low nutrient levels become red).
   (d) Activity (cells not actively growing are red).
   (e) Age (old cells are red).

Figure 2. The tools: toothpicks and agar plates.

for a week. A list of the typical precautions can be found in Table 2; additional hints are available on-line (White 1999; Manney 1997b). The students should also be reminded to take careful notes of what they do. They then perform the experiments and leave them for a week at room temperature. In some cases, they may find it useful to observe their samples as they grow.

Figure 3 shows some typical experiments and results. In general, red transferred to new medium grows up to give a mixture of red and white while white gives only white. However, red and white usually retain their colors when starved of nutrients. In addition, students often come up with very creative experiments including: placing red or white on an "island" of fresh agar, placing a piece of fresh agar on top of samples of red and white, mixing red and white, etc. (not shown). Virtually any experiment the students can carry out will yield useful information and lead to constructive discussion.

Table 2. Precautions for avoiding contamination.

1. Before starting to work, remove all extra material from the lab tables and wipe the tabletop with alcohol.
2. Tie back your long hair and roll up your sleeves.
3. Avoid too much bustling in the lab.
4. When you must open your plates, open them for only the minimum time necessary.
5. Do not touch the nutrient medium in the plates with your fingers or anything except a toothpick.
6. Take toothpicks out of the tubes carefully and hold the toothpick only by the end that will not be touching the yeast or the plates.
7. If you even *think* that you've contaminated the toothpick, get a new one.
8. Do more than one replicate of each experiment and do the replicates on different plates. Put each set of replicates in a different bag.

The second session (one week later) begins with discussion of the results of their first round of experiments. As in the first session, the students begin discussion in their groups and then continue as a class. While the first session consisted mostly of brainstorming, the second session is more data-focused and rigorous. Throughout the discussion, the teacher takes the role of moderator; it is essential that the ideas and evaluation come from the students. As a result of this conversation, the students often are able to rule out some hypotheses and answer some questions; however, many disagreements often remain.

After the discussion, students plan, present and carry out their second round of experiments. These are more carefully focused than the first round as the students come to see the importance of details they had overlooked previously or neglected to record in their lab notebooks.

The third and final day is devoted to a discussion of their results and an attempt to reach a consensus about what they have found. These discussions, as well as those that take place during the second session, are the most critical part of the experience. They are student-driven, but not "anything goes"; hypotheses are evaluated in the light of the data that the students have collected. Although the conversations often have a similar overall course, they are very different from section to section and from year to year.

Student Discussions

During the discussions, students frequently disagree about the details of their results. For example, almost all groups place a sample of white by itself on fresh medium. While most of these samples grow up to give pure white, on occasion some replicates show a slight red tinge. This provokes extensive discussion about whose results are right. After much debate, the students usually decide to resolve this by trying multiple replicates in round two and by paying closer attention to how they prepare these replicates.

There are also frequent disagreements over interpretation of results. As an example, one group focused on the finding that red *or* white placed on the lid to starve retained its original color. From this, they concluded that the difference between red and white could not be due to starvation, since very dramatic starvation did not cause any color change. Others were unmoved by this and argued that dying quickly was different than gradually starving in the presence of limited nutrients. As in research, disagreements like these have no simple resolution; they usually persist until the results of other experiments shift the balance.

Having done this many times, I have found that it is important to trust the students to take the discussion into areas they find productive. Many times, I have been dragged off on what seemed to me to be unproductive tangents only to find later that the students saw these as essential. The degree of guidance I offer varies—with very inexperienced students, I try to point them towards questions they can answer with the tools they are given. With more advanced students, I try to see that each strand of the discussion is thoroughly explored. In either case, I let the students go in the direction they find most compelling. The point is to explore the students' ideas in their own terms, rather than to find the answer by a predetermined path.

What They Can Find Out

From these and other experiments, the students show that there is more red and more white after a week's growth than at the start—therefore, both red and white are alive, and both red and white can grow. They demonstrate that large white samples do not have a red center—therefore, the yeast is not a single strain whose color depends on environment. They also find that red gives rise to red and white cells while white only gives rise to white—this is the "tip of the iceberg" of the answer. In almost all cases, by the end of the last session, students reach these conclusions—often by very different paths.

I have also used this exercise successfully with middle school students. Although they require more guidance, their theories, experiments and findings were remarkably similar to those of the college students. In smaller classes like these, students could be

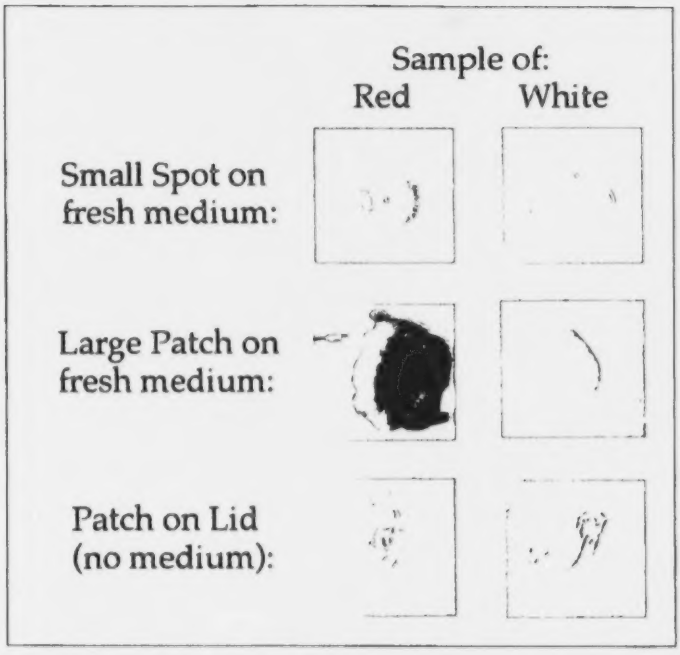

Figure 3. Some typical experiments and results.

asked to keep journals and to respond in writing to their classmates' work to further model the process of research.

Connections to the Rest of the Course

In General Biology I at U Mass Boston, this lab is part of a larger effort to make the process of science clear to the students. My first lecture is devoted to describing how a commonly accepted scientific fact, that smoking causes cancer, came to be. I talk about initial hypotheses, ruling out alternative models, and how the accumulation of evidence leads to consensus but not certainty. I then explain that their first lab, this yeast experiment, is designed for them to experience this process first hand.

As the lab progresses, I take time in lecture to highlight important issues that their discussions raise as well as to connect their experiences in the lab with the development of the scientific ideas in the course. I make the point that science is a problem-solving activity and that hypothesis and experiment interweave and develop together in research just as it did in their lab.

I use their disagreements over their results to show how results are not always clear-cut and how a scientific community deals with discrepancies. I ask them to consider the following questions: Should the majority decide what the correct result is? What constitutes an identical replication? How can we decide which data to disregard? How could you design an experiment to distinguish between these possibilities?

Their disagreements over interpretation allow me to make another important point about scientific knowledge: like members of any scientific community, they each make up their own minds about what is going on. Debates rage for years about interpretation, and no one experiment convinces everyone. The consensus is what makes it into the textbook. In this way, the issues raised in the lab echo throughout the semester.

"The Answer" & Its Role in the Classroom

Although I play down the right answer and many students are satisfied with what they've found, some insist on finding out exactly what's going on. For those who ask, I give the following explanation: The cells start off containing a plasmid; cells with the plasmid are red, cells without the plasmid are white. The plasmid contains a gene that encodes an enzyme involved in the synthesis of adenosine, a precursor of DNA and RNA. This enzyme converts a colorless precursor into a compound that becomes red on exposure to oxygen.

A cell with the plasmid usually buds off a daughter with the plasmid, thus one red cell yields two red cells. However, the plasmid is unstable; because it lacks a centromere, one copy is not always segregated into each of the daughter cells. As a result, about 1% of the time, the plasmid is not transferred to the bud; in this case, one red cell gives rise to a red cell and a white cell. A daughter cell without the plasmid cannot regain it. Therefore, cells without the plasmid can only bud off daughters without the plasmid; thus one white cell always yields two white cells. This gives rise to the pattern that the students observe: Red gives red and white while white gives only white.

Because cells can only lose the plasmid, as the culture grows, the fraction of cells that are red gradually decreases. In addition, at the edge of the patch, the cells have more access to nutrients and therefore grow faster. Therefore, after a week there have been more cell divisions on the edge, therefore more chances to lose the plasmid—so the edge of the patch is white. On the other hand, the cells in the center of the patch are surrounded by other cells, have less access to nutrients, and therefore grow slower. Since they have undergone fewer divisions, a higher fraction of the cells in the center portion of the patch are red.

The students often find this explanation unsatisfying. The connection between what they can figure out and the pattern of the patch is lengthy and complex. They often protest (correctly) that they "could not have found plasmids with a toothpick!" Because of this, I do not make finding and giving the answer an important part of the exercise. Instead, I ask that their lab reports describe some intersection of experiment and hypothesis: what they thought, what they did, and how their results changed what they thought. As a result, their reports allow me to observe and comment on their thought processes in detail.

Student Evaluations

At the end of the General Biology course, two months after they had completed this experiment, I gave my students an anonymous free-response survey asking them what they thought of the "red and white yeast lab." The results were encouraging; positive responses outnumbered negative responses 2 to 1. The majority of the complaints centered around not getting the answer or knowing that they couldn't find the whole answer with

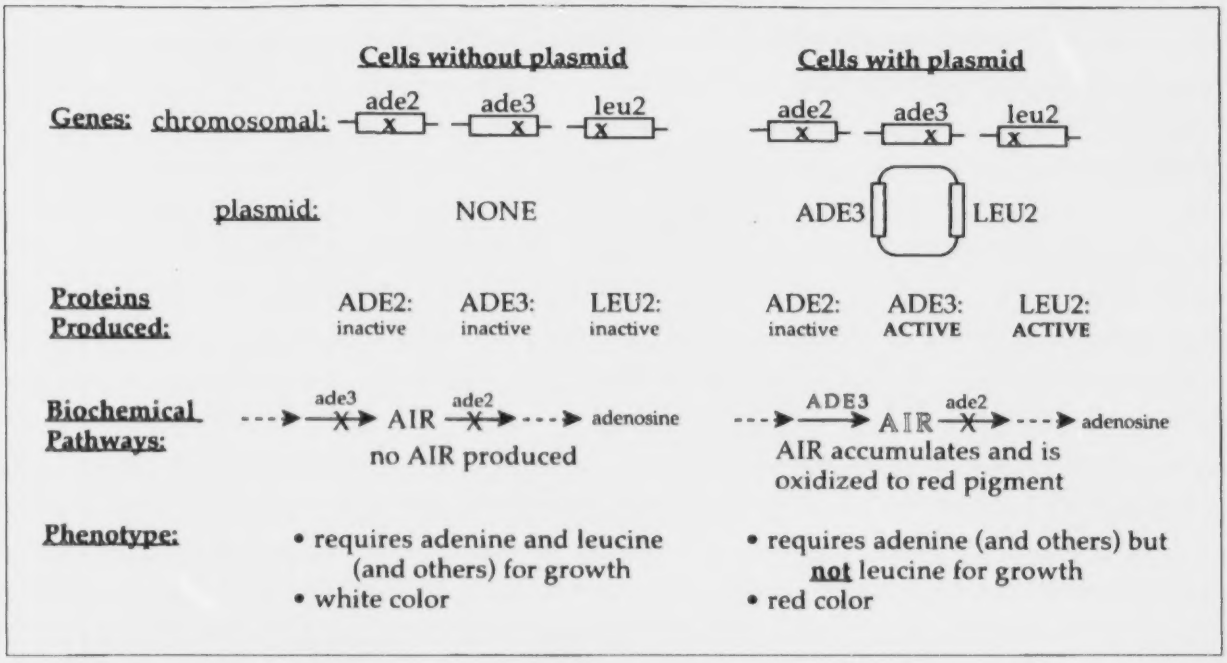

Figure 4. Genetics and biochemistry of yeast strains with and without the plasmid. Genotypes in lower case indicate inactive recessive mutant alleles; "x"s indicate the sites of mutations and respective biochemical blocks. Genotypes in all capitals indicate functional wild-type alleles; the intermediates produced are shown in outline type. Note that both strains also require uracil, tryptophan, lysine and histidine.

the tools they were given. One comment typical of these was, "It p....d me off, I want to know the real story." On the positive side, many students mentioned that they enjoyed designing their own experiments and sharing their ideas; they reported learning about designing and testing hypotheses and the scientific method. One wrote, "I liked not knowing what would happen. It 'opened your mind' a lot more and made you think."

Materials & Methods

Detailed techniques for making the media and tools required can be found in the Appendix as well as on-line in my lab manual (White 1997) or in an on-line manual of yeast lab techniques (Manney 1997b).

The yeast strain has the genotype: α ade2 ade3 ura3 leu2 trp1 lys1 with the plasmid pDK255 that carries LEU2 and ADE3. It is available from the ATCC (Accession 201926) or through Carolina Biological Supply Catalog #15-6249. In standard yeast notation, alleles in lower case indicate a recessive mutant phenotype; those in upper case indicate a dominant wild-type phenotype. ade3 strains are blocked in the synthesis of adenine nucleosides before the formation of the compound 5-amino imidazole ribonucleotide (AIR); ade2 strains are blocked immediately after the production of AIR. As a result, the cells have the phenotype of ade2 ade3 without the plasmid, and no AIR builds up. Because the plasmid carries a dominant wild-type allele of the ADE3 gene, cells with the plasmid are phenotypically ade2 ADE3 so that AIR accumulates and is oxidized to a red pigment (Manney 1997c). This is shown in Figure 4.

Two types of sterile medium in petri plates are required for this experiment. A small number of CSM-leu plates are required to prepare the initial patch. Cells without the plasmid are leu2 and thus require leucine for growth. Because the plasmid carries a dominant wild-type allele of LEU2, cells with the plasmid are phenotypically LEU2 and do not require leucine for growth. CSM-leu medium contains all essential nutrients except leucine and thus only allows cells carrying the plasmid to grow (see Figure 4). The strain should be stored on CSM-leu plates between uses. YPD medium is nonselective (cells with or without the plasmid will grow) and it supports the production of red pigment. YPD medium is used for the initial patch and all the students' experiments. Each group of students will need 6 to 10 plates of YPD medium for each round of experiments.

Sterile toothpicks are required to take samples; the round ones with pointed ends work best. Sterile water and a few sterile test tubes are also required to make the yeast suspension. Bags for the plates, markers, tape, and access to an autoclave are also necessary.

Preparation for Lab

Two weeks before the lab is to start, streak out the strain on CSM-leu plates. Let it grow for one week at room temperature. Then scoop up one large colony or a few small ones (a 1-mm sphere is sufficient; the exact amount is not critical). Resuspend this in 1 ml of sterile water in a sterile tube. Then spread 30 to 50 µl of the suspension into a roughly 2 × 4 cm patch on each of two YPD plates for each group. I have used a micropipet with sterile tips for this; sterile Q-Tips™ also work well. The patches then grow for a week at room temperature and are ready on the first day of the lab. Plates should be incubated with the lids down to prevent drops of condensation from falling on the agar.

Acknowledgments

I would like to thank Julie Archer, Jeanne Bamberger and Frank Solomon for their help in developing this lab; without them, this would not have been possible.

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Appendix

Media Recipes & Other Instructions

CSM-leu Medium

43.7g DOBA Medium (Bio 101 #4026-012)
0.69g CSM-LEU Supplements (Bio 101 #4510-512)
Add water to make 1 liter and autoclave.

For details, see the Bio 101 Web site: http://www.bio101.com.

YPD Medium

10g Bacto Yeast Extract
20g Bacto Peptone
20g Bacto Agar
Add water to make 1 liter and autoclave.
Add 50 ml sterile 40% glucose.

These ingredients are available from a variety of scientific supply outlets.

Pouring Plates

Each plate takes roughly 25 ml medium. Contamination is often a problem, so YPD plates should be poured in a sterile hood or under very clean, draft-free conditions. The plates should also be allowed to dry with their lids on and stored at room temperature to allow contamination to become visible; contaminated plates should be discarded. Empty sterile plastic petri dishes are available from many scientific supply outlets; 100 × 15 mm work best.

Sterilization

Media should be sterilized in Erlenmeyer flasks having at least twice the volume of the medium being sterilized; they should be capped with tinfoil. Toothpicks can be sterilized in test tubes with caps or wrapped in foil. Autoclave for 15 minutes.

Alternative Media and Preparation

YEP medium can be substituted for YPD medium with no difference in the behavior of the strain. Media can also be sterilized in a pressure cooker. Details of YEP preparation and pressure-cooker sterilization can be found at the GENE project's Yeast Web site (Manney 1997b). CSM-leu medium is also available from Carolina Biological Supply: pre-poured plates are from Catalog #82-1997; bottles of media are from Catalog #77-6750. Pre-poured YPD and CSM-leu plates are also available from Bio101.

Cells & Molecules

Making pH Tangible

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The concept of pH is an important one for our students. All of our experiments depend upon pH, and nearly every process in living cells depends upon the maintenance of a proper pH level, as enzymes will generally only function within a narrow pH range. Since the synthesis of adenosine triphosphate in living cells depends upon the creation and maintenance of a pH gradient, it is worth noting that nearly all of the energy used by all living systems on this planet comes from pH gradients.

Many students have a difficult time understanding the concept of pH, as it is somewhat abstract, and mathematical on a logarithmic scale. We present here a simple set of experiments to give students practical, hands-on experience with pH. The labs also touch on the mathematics by demonstrating the logarithmic nature of the scale. These experiments are appropriate for high school biology or introductory college-level biology labs.

pH is the measure of the hydrogen ion (H^+) concentration of an aqueous solution. In our experiments, as well as within our bodies, we keep the concentration of H^+ ions constant by using buffers, chemicals that will compensate for changes in this ion by absorbing excess ions as the pH goes down, or freeing them as the pH goes up. For instance, our blood must maintain a pH level of 7.35–7.45. A slight change can cause severe illness and even death. Therefore blood must have a system with which to control the level of pH. One such system is a bicarbonate buffer system, which consists of carbonic acid, a weak acid, and sodium bicarbonate, its complementary weak base.

$$H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$$

When the concentration of H^+ ions increases, they are removed with the formation of carbonic acid. When the concentration of H^+ ions decreases, they are replaced by the dissociation of carbonic acid molecules. Therefore, the concentration of H^+ ions remains constant, maintaining this range of pH[1].

In this lab, students will test the pH of different substances: acids at various concentrations to demonstrate the logarithmic nature of the scale, and common household substances. They will also study the effect of a buffer on acidic solutions by comparing the behavior of buffered and unbuffered solutions upon the addition of acid. Finally, they will compare common over-the-counter antacid remedies.

Procedure

1. pH of Different Concentrations of Acid

If time allows, students should prepare the following concentrations of acid from 0.1 M HCl stock.

0.01 M HCl
1.0 mM HCl
10 micromolar HCl
1.0 micromolar HCl
Distilled water

Instruct the students to calculate the expected pH of each of these solutions, using the formula $\mathbf{pH = -log[H^+]}$ by plugging the concentration of acid into the equation. Remind the students that in addition to the HCl, the water in aqueous solutions contributes 10^{-7}M H^+. For example, for 10 mM HCl:

$\mathbf{10\ mM = 10^{-2}M}$. Thus,
$\mathbf{pH = -log[10^{-2}] = 2}$.

Use narrow range pH paper to determine the pH of each solution. Record. There will be some variation from the expected pH of each solution, due largely to the inaccuracy of the pH paper. However, all results should be within 1 pH unit of calculated expectations.

2. pH of Foods & Household Substances

Have the students predict the pH of each of the following substances, and then test with pH paper:

Vinegar
Lemon juice
White grape juice
Cola (clear, preferably)
Tap water
Distilled water
Milk (diluted with 2 parts distilled water to decrease any interference from the white color)
1% Drano®

For example, the sour taste of vinegar is attributed to acetic acid; thus, vinegar may be expected to have a pH of below 7.

Each student should bring in one additional household substance for testing.

3. Buffers

This procedure tests how distilled water, NaCl, and a sodium phosphate buffer solution react to the addition of HCl. Sodium phosphate is chosen because it is a fairly strong buffer and is commonly used in biological experiments. It is also simple to explain its buffering capacity, as the phosphate ion may exist in solution with zero, one, two, or even three H^+ ions attached:

$$[PO_4]^{3-} + H^+ \leftrightarrow H[PO_4]^{2-}$$

$$H[PO_4]^{2-} + H^+ \leftrightarrow H_2[PO_4]^-$$

$$H_2[PO_4]^- + H^+ \leftrightarrow H_3[PO_4]$$

The pH indicator Brom Cresol Purple is used to detect a pH change. This indicator should initially be purple and will change to yellow at or below pH 5. You may substitute chlorophenol red, or methyl purple if you prefer.

Prepare 0.1 M sodium phosphate solution, 0.1 M NaCl, and 0.1 M HCl as described in Table 1.

Each student or group should obtain three test tubes, labeled for 0.1 M sodium phosphate buffer, distilled water, and 0.1 M NaCl. 5 mL of the appropriate solution should then be dispensed into each tube.

Add 3–4 drops of indicator dye to each tube and mix until homogeneous.

Test each tube by adding 0.1 M HCl dropwise, mixing the solution after each drop. Continue to add acid until the solution changes color, indicating that the solution has become acidic. Record the number of drops of acid needed to generate the color change. Students should see that acid added to water or NaCl immediately changes the pH of these solutions rather significantly; however it takes quite a bit of acid to change the pH of the buffered solution.

Table 1. Solutions and reagents.

To prepare 0.1 M phosphate buffer solution:
Prepare 0.1 M Na_2HPO_4 by dissolving 14.2 g anhydrous Na_2HPO_4 in a liter of distilled water.
Prepare 0.1 M NaH_2PO_4 by dissolving 12.0 g anhydrous NaH_2PO_4 in a liter of distilled water.
Mix 3.5 parts 0.1 M Na_2HPO_4 with 1 part 0.1 M NaH_2PO_4.
Use dilute NaOH or dilute HCl [such as 10 mM] to adjust the pH to 7.0.

To prepare 0.1 M NaCl:
Dissolve 5.8 g NaCl in a liter of distilled water.
The solution should be pH 7, but for this lab, the pH should be checked. If it is more than 0.1 units off, use dilute NaOH or dilute HCl [such as 10mM] to adjust the pH to 7.0.

To prepare 0.1 M HCl:
Most commercial preparations of concentrated HCl are 12 M. Thus, to prepare a 0.1 M solution, add 8.3 ml concentrated HCl to 991.7 ml concentrated HCl.
Note: Distilled water used in the buffers section should also be brought to pH 7.0 using dilute NaOH or dilute HCl [such as 10 mM] before use.

Brom Cresol Purple may be purchased from Fisher Chemicals, catalog #LC11890-7.

4. Commercial Antacids

This procedure is designed to test the buffering effects of different commercial antacids. That is, it will test how well they can absorb excess H^+ ions, thus "neutralize stomach acid." The stomach produces HCl; therefore, it is the acid of choice for this experiment. Again, Brom Cresol Purple is used as a color indicator to show when the buffer is no longer neutralizing the acid.

The following products should be tested:

Rolaids®
Tums®
Maalox®
Alka-Seltzer®
Distilled water

A tube should be labeled for each of the products listed above. Each student or group can prepare each product, or the instructor may prepare them in advance. Crush the amount needed for one dose of each medication and dilute each with distilled water to 100 mL. Some of the products may require extensive mixing to get most or all of the powder into solution.

Pipet 5 mL of the dissolved products into the appropriate tubes. Add about 4 drops of Brom Cresol Purple to each tube and mix until homogeneous.

Add 0.1 M HCl dropwise to one tube at a time and mix after each drop. Continue this process until the solution turns yellow, indicating an acidic solution. Record the number of drops of acid needed to generate the color change.

You should find that Alka-Seltzer absorbs the most acid, and Tums places second. You might want to explain to the students, however, that Alka-Seltzer contains sodium bicarbonate, while Tums consists of calcium carbonate. Thus, although Alka-Seltzer is most effective, people on sodium-restricted diets should avoid this and turn to Tums instead.

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Using Apple Peel Sections To Study Plant Cells & Water Relations

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The thin leaves of the waterweed, *Elodea*, and the epidermal peels of onion, *Allium*, have provided the "first look" at microscopic plant cells for biology and botany students for many decades (Morholt & Brandwein 1986). Both sources are readily available, thin enough so that students do not need to slice or section the tissue, and together, provide easily recognizable cellular structures. *Elodea* provides a good microscopic introduction to the spatial arrangement of green plant cells, the properties of the cell wall and cell membrane, cytoplasmic streaming, and cellular response to plasmolysis under hypertonic conditions. Onion epidermal tissue allows students to study the nucleus, nucleolus, and other cytoplasmic features. Purple onion provides an opportunity to study both the common cellular and cytoplasmic structures typical of epidermal cells of white onion, and affords the additional advantage of observing vacuolar anthocyanin pigments (Kamrin & LaVan 1984).

In this article, we suggest a third plant species which can further enhance the plant cell laboratory; namely, the cells of an apple peel. When combined with *Elodea* leaves and onion peels, apple peel cells add yet another dimension to the composite model that students are challenged to develop as they study plant cells.

First, we shall describe the structure of apple peel cells, and the benefits of including them in the study of plant cells. Then, we will suggest a number of questions to stimulate further investigations for open-ended laboratories or independent studies.

Discovery

We discovered the potential of apple (*Malus domestica*, variety 'Red Delicious') peel cells during a botany laboratory being taught with an "inquiry approach." The inquiry approach as defined in a recent report, *Developing Biological Literacy* (1993), is "a method that combines laboratory activities with student-centered discussion and discovery of concepts." In our laboratory, students were instructed to perform a comparative study of the cells of *Elodea* leaf, purple onion, potato parenchyma, and red bell pepper (*Capsicum*). Because it was uncertain whether it would be possible to secure a purple onion, we also brought a beautiful Red Delicious apple to the laboratory.

During the laboratory, some students chose the apple and proceeded to prepare thin sections of peel tissue instead of the purple onion. What a delightful discovery they made! Needless to say, we were also excited to see not only the beautiful, red, anthocyanin pigments but the vacuolar membrane (tonoplast) visible as a distinct boundary which separates the pigmented vacuolar sap from the surrounding cytoplasm. In addition, most of the distinct cellular features of onion and *Elodea* are also present, namely, cell walls, plastids, and possibly nuclei.

Materials

Apple ('Red Delicious' or other red variety) sliced into quarters
Sharp, single-edged razor blade
Microscope slide and coverslip
Spatula
Dropper bottles: Distilled H_2O, 10% NaCl, 0.1 M HCl, 0.1 M NaOH
Compound microscope

Procedure

The procedure for preparing free-hand sections of apple peel cells is fairly straightforward.

1. Obtain a clean microscope slide and coverslip. Add a small drop of distilled water from the dropper bottle to the microscope slide.
2. Select a quarter of an apple with the peel intact. Firmly hold the apple slice with one hand and a sharp razor blade between the thumb and index finger of the other hand. Rest both hands on the laboratory table so as to protect your fingers, your body, and your neighbor from injury.
3. Draw the blade across the surface of the peel tangentially so as to obtain very thin, almost transparent sections. Pull the blade lengthwise during the cutting motion so as to use as much of the blade surface as possible. With spatula, tip the sections into the drop of water on the microscope slide. Use different locations on the peel surface to obtain several sections. Some practice and patience are required to obtain thin sections.
4. When you have several promising peel sections, add the coverslip by first touching one edge to the microscope slide near the drop and then lowering the slip over the drop in hinge-like motion. Use the low power of a compound microscope to locate a thin section with some intact cells. Cells tend to be thinnest near the perimeter of the sections. Center the thinnest intact cells within the microscopic field of view before examining them under the high power objective.

Apple Peel Cells Under Magnification

Figure 1 illustrates the appearance of apple peel cells under a magnification of 400×. Unlike *Elodea* leaves and onion epidermis, apple peel offers students the benefit of seeing a clearer distinction among compartments within the cells. The vacuole, bounded by the tonoplast (vacuolar membrane), is easily located because it contains colorful anthocyanin pigments. The tonoplast separates the vacuolar compartment from the surrounding cytoplasm. Plastids, granular inclusions, and possibly membrane fragments or vesicles can be seen in the cytoplasmic region surrounding the vacuole (personal communication, Goffinet 1995) (Figures 1 and 2).

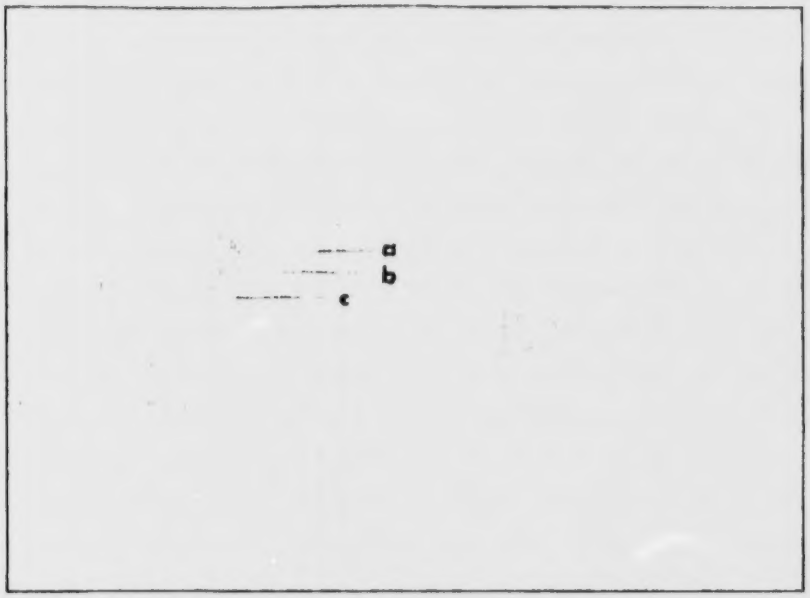

Figure 1. Magnified apple peel cells (var. 'Red Delicious') showing cell wall (a) and cytoplasmic region (b) surrounding a large vacuole (c). 400×

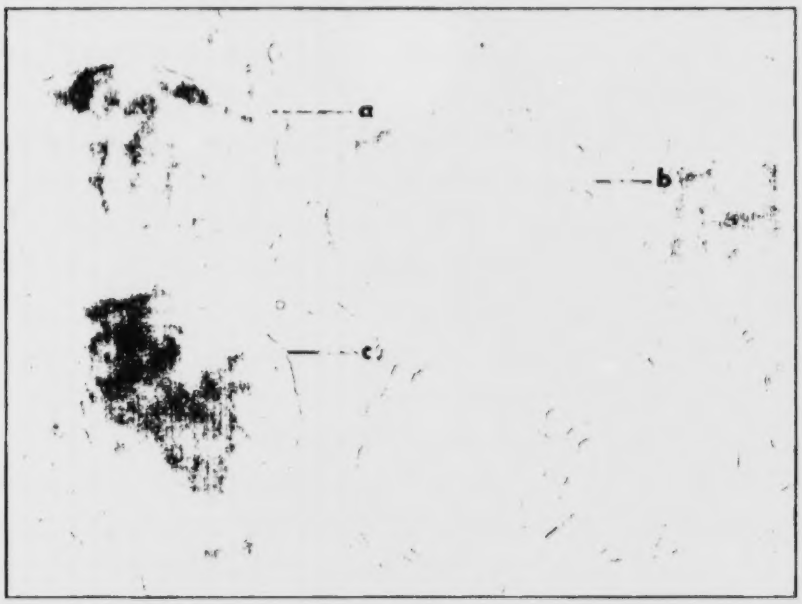

Figure 2. Apple peel cells (var. 'Red Delicious') showing cell wall (a), cytoplasmic region (b), and tonoplast (c). 1000×

The distinct vacuole of apple peel cells also affords the opportunity to study plant cell water relationships. Plasmolysis is the shrinkage of the vacuole and cytoplasm that results from a loss of turgor pressure as water diffuses outward across the tonoplast and plasmalemma. Water diffuses out of the vacuole and cytoplasm when the aqueous medium surrounding the apple peel sections is replaced by a hypertonic medium such as 10% NaCl. Water can be drawn from beneath the coverslip by placing a dry paper towel against one edge of the coverslip. At the same time, 10% NaCl is added to the opposite edge of the coverslip with a dropper pipet so that hypertonic (salt) solution displaces the water. After several minutes, the shrinkage of the vacuole can be observed (compare Figures 2 and 3). Unlike *Elodea* and onion peel cells, apple peel cells afford students the opportunity to observe the effects of plasmolysis in both the vacuole and cytoplasm.

Additional Inquiry Into Apples

Because apples are so much a part of our everyday lives, their use in plant cell investigations can initiate a host of additional relevant investigations. In keeping with an inquiry approach, students should be invited to develop questions and hypotheses for further study of apple peel cells. The discussion should focus first upon student *observations* (e.g. presence of pigment). Then, students can be challenged to relate each observation to its biological *significance* (e.g. relationship of color to fruit ripening). For example, we have noticed that a greater average duration of time is required to induce visible plasmolysis in apple peel cells than in *Elodea* or onion peel cells. One hypothesis to account for this is that the cell wall (i.e. apoplast) of apple peel cells has a higher resistance to water movement perhaps due to impregnation with a waxy or other hydrophobic component. Such a property, in conjunction with the epidermal cuticle, would serve to resist desiccation. If this hypothesis is correct, why would such an adaptation be more essential in apple peel cells (dry, atmospheric environment) than in *Elodea* (aquatic hydrophyte) or onion epidermal cells (subterranean environment)? Thus, students learn to relate cellular morphology and physiology to the natural environments of these plant organs.

The subject of plant pigments and fruit ripening was explored during a discussion with students. Some of the questions in Table 1 resulted from this discussion, and the references cited were located in a subsequent literature search.

In particular, the properties of anthocyanins can be investigated. If both red bell pepper (*Capsicum*) and red apples are included in cell structural studies, students can investigate differences in cellular location of the red color and correlate location (plastids *versus* vacuole) with water solubility.

Experimental evidence that the red color of apples is truly anthocyanin and not betalain pigment can be obtained while also observing the effects of vacuolar pH on pigment

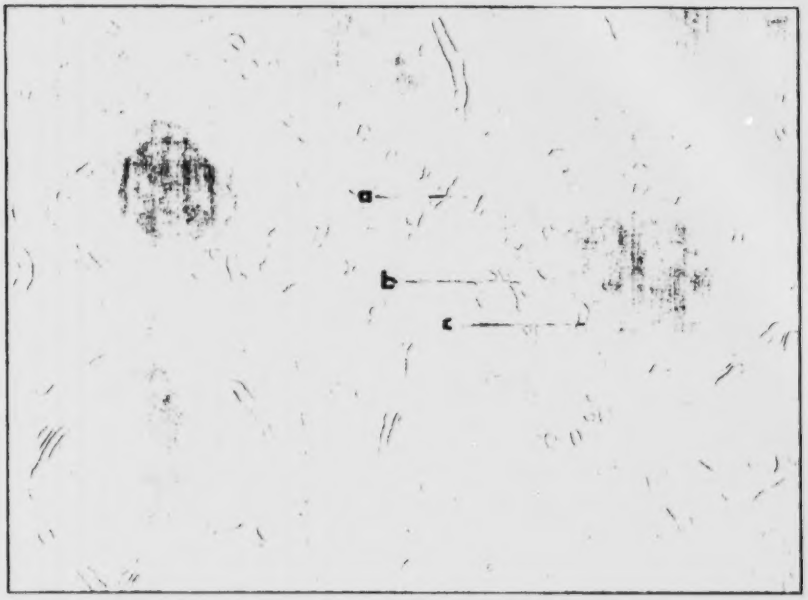

Figure 3. Plasmolyzed apple peel cells (var. 'Red Delicious') showing cell wall (a), cytoplasm (b), and vacuole (c). 1000×

Table 1. Questions leading to further investigation of apple peel cells.

1. What changes in pigmentation occur in the plastids and vacuoles of apples when the fruit changes color from green to red during ripening? (See Blanpied 1982.)
2. How do the changes in color noted in #1 differ between red apple (*Malus*) and red bell pepper (*Capsicum*)?
3. What is the approximate pH of the vacuolar fluid? (See Lancaster et al. 1994.)
4. Are apples red because of the presence of anthocyanins or betalains? (See Moore & Vodopich 1987.)
5. Do different apple varieties (or home-grown *versus* store-bought; or immature *versus* ripened) differ in cuticle thickness? Cellular properties? Tendency toward plasmolysis?

color. Apple peel sections can be exposed sequentially to 0.1 M NaOH and 0.1 M HCl using the same procedure as described above for NaCl treatment. The change from red to blue color during exposure to NaOH provides evidence that the red pigments of apple peel vacuoles are anthocyanins because these pigments, unlike the betalains (e.g. in red beet roots), do change color in response to pH changes (Moore & Vodopich 1987).

We propose that apple peel cells be included in laboratories aimed at plant cell investigation. These cells provide an important additional dimension to help students formulate a concept of the plant cell structure and function. In addition, apples are a "fruitful" resource to stimulate more advanced, open-ended experimentation.

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The Giant Cell

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Do you remember how difficult it was to *truly* understand how the parts of the cell related to one another? As your teacher repeatedly discussed the cell and its parts with you, trying to introduce you to an understanding of the relationship of the parts to one another, it was as though he or she had "been there" on a fantastic journey and was trying to help you "see" what he or she had seen. Frequently in situations like this words fail both the student and the teacher. This paper presents a method for the teacher to take students inside a cell.

One of the more difficult tasks for a teacher is to convey a concept or mental image just using words to accomplish the task. Biology students at Asheville High School are learning about the cell by visiting a cell, going inside it, looking at it, and touching its various parts to see how they relate to each other and work together so the whole cell can function and survive. In two and a half weeks students construct a cell 20 feet long, 10 feet tall, and 10 feet wide, complete with all organelles. Students of all abilities can complete the project in this amount of time. Materials used include ordinary household items and an extremely large sheet of thick garbage bag plastic. All organelles are accurately shaped and proportionally sized. They are hung from the top of the cell by invisible monofilament line. The cell constructed is "generic" in nature. Without chloroplasts it is an animal cell with cell membrane. When the chloroplasts are inserted, it becomes a plant cell. Student guides point out to visitors that the rigid cellulose cell wall is missing.

When the cell is inflated (by a window fan, see Figure 1) the students go *inside*, sit on the floor, walk around, discuss all the parts, see relationships, and touch organelles in order to better understand and appreciate how they function together as an organized structure. In preparation for the "mother of all tests" or "the easiest test in the history of the world" (depending on whether you are talking to the teacher or the student), the students spend several class periods inside the cell discussing and explaining to each other and students from other classes who visit the cell just how all the parts work to contribute to the success of the cell.

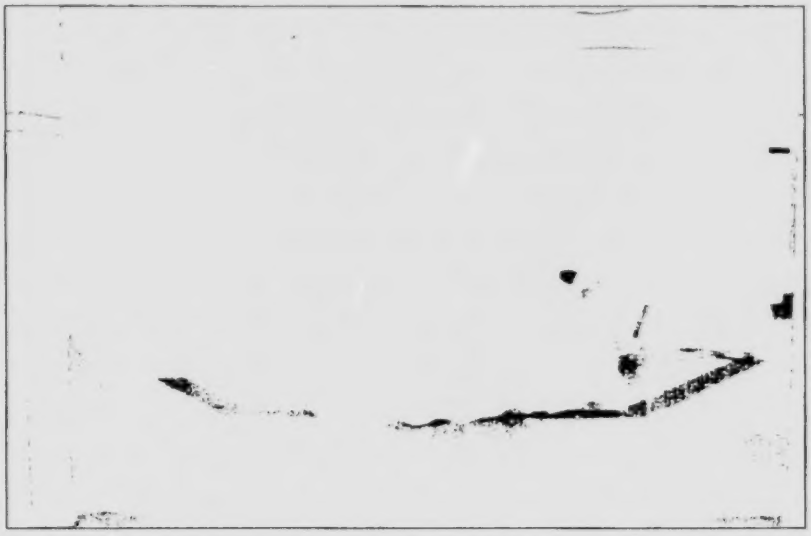

Figure 1. The inflated cell with entrance tunnel. Student on the right holds the window fan used for inflation.

The experience gained by guiding other students on tours inside the cell turns the students into expert "presenter/guides." Having acquired that experience, the students quickly ask about taking the cell "on the road." When the students mention this in class most teachers immediately think of places such as local elementary and middle schools, and local and regional science museums. The students immediately bring teachers back to the true vision of what taking the cell on the road really means: trips to display it at regional or national theme parks.

Total cost for the experience: less than $175.

Construction Details

The following is a brief description of how to construct the cell. The reader is encouraged to study the photographs that accompany this article. Construction is a three-step process:

1. Build the cell.
2. Build the organelles.
3. Install the organelles.

Additional detailed drawings, descriptions, and a list of supplies and suppliers are available from the author at no cost.

The Cell Membrane

Cut 10′ off the end of a 50′ × 20′ sheet of 6 mil plastic (see Figure 2). Cut this smaller 10′ × 20′ sheet into two 10′ squares. These are the ends of the cell. Tape them to a 40′ × 20′ sheet to make a rectangular box measuring 10′ × 10′ × 20′. The students do this more easily if they first make and tape a paper model 2″ × 2″ × 4″ in size. Tape each seam on the large cell with 2-inch wide clear tape, and overlap each edge of the tape with an additional piece of tape.

The Openings in the Cell Membrane

Cut a 4′ × 5′ opening in the long side of the cell, near one end. Construct a 4′ × 5′ × 10′ tunnel and tape it to the opening (see Figure 3). People enter and leave through this. The end rolls up to make it air tight.

Near the other end of the cell cut an opening to hold a window "box"

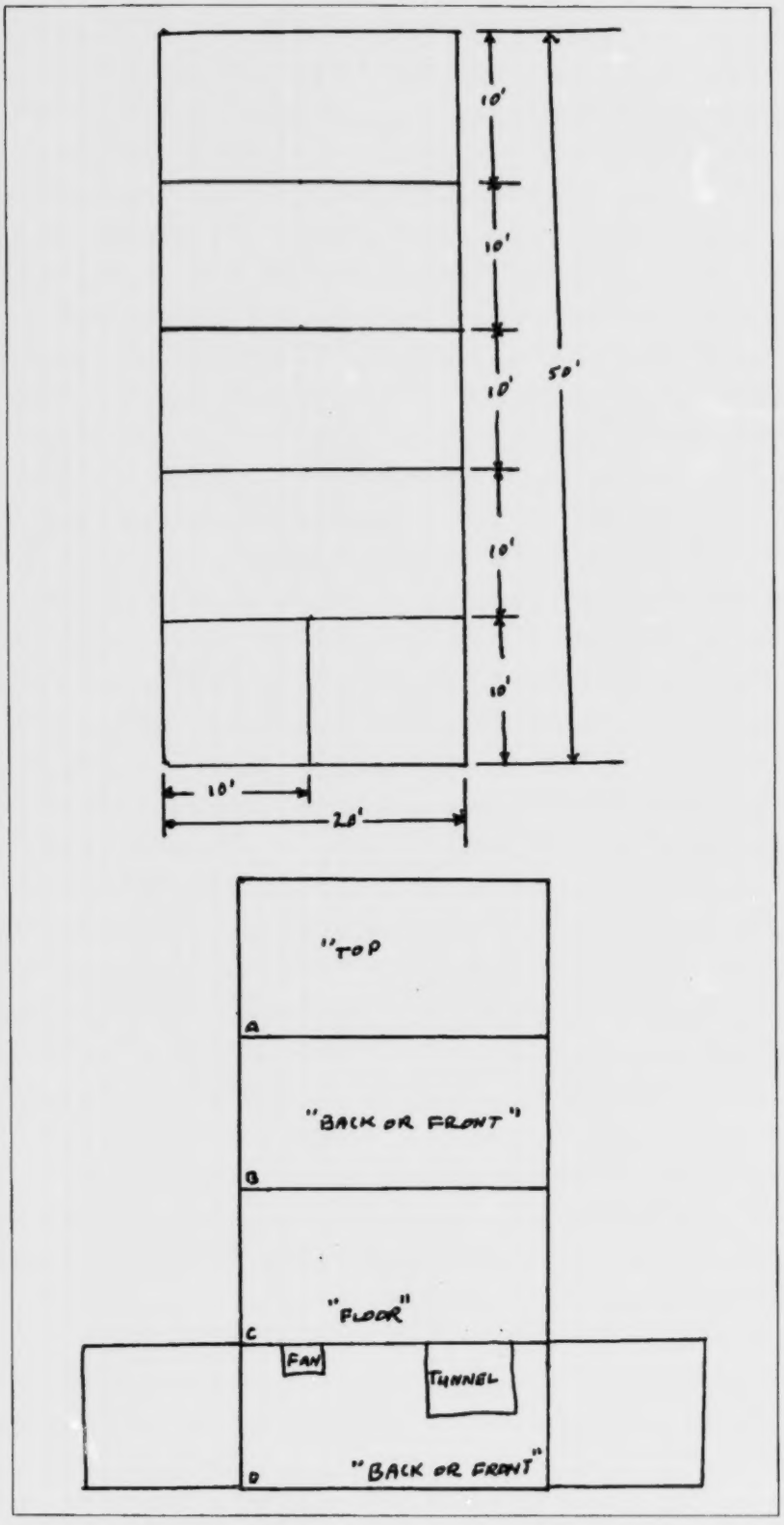

Figure 2. Construction of the cell membrane.

fan. The fan sits on the floor. Several pieces of tape will hold it to the cell.

Ribosome

Cut a ping pong ball in half. Glue each half to 2 ping pong balls. This makes 2 ribosomes. Make about 50 of them. Paint them a bright color.

Nucleolus

Inflate a round balloon to a diameter of 8 to 10 inches. Papier-mâché half of it. When dry, remove the balloon and paint it. Glue a ribosome to its outer surface (see Figure 4).

Nucleus

Fill a small trash bag with packing peanuts. Papier-mâché the bottom half of the bag. When dry, remove the peanuts and the bag. Paint the inside with flat black paint. Paint the outside gray, with occasional black dots to represent nuclear pores. Use a hole punch or nail to make 2 or 3 real pores.

Mitochondria

Fill a bread bag with "packing peanuts" or crinkled newspaper. Cover with papier-mâché. When dry, empty the peanuts and pull out the plastic bag. Cut the papier-mâché shell lengthwise to make 2 longitudinal sections of a mitochondria (see Figure 5). Make 6 or 7 so you will have a total of 12 to 14 mitochondria to put in the cell. Paint them inside and outside.

Cut accordion style folded circles to make the cristae. Put puff paint dots on the cristae to represent the enzymes found on its surface. When dry, use a hot glue gun to glue the cristae "accordion style" inside the mitochondria. Make a dozen or more mitochondria.

Golgi Apparatus

Staple paper plates back to back. Separate pairs of stapled plates with rings cut from a round oatmeal box. Spray paint and tape several balloons to the edge to represent vesicles budding from the Golgi (see Figures 6 & 7).

Vacuoles

Inflate 2 to 3 balloons to a diameter of 10" to 12". Papier-mâché. Remove the balloon and cut in half. Spray paint. Cover open side with clear transparent film.

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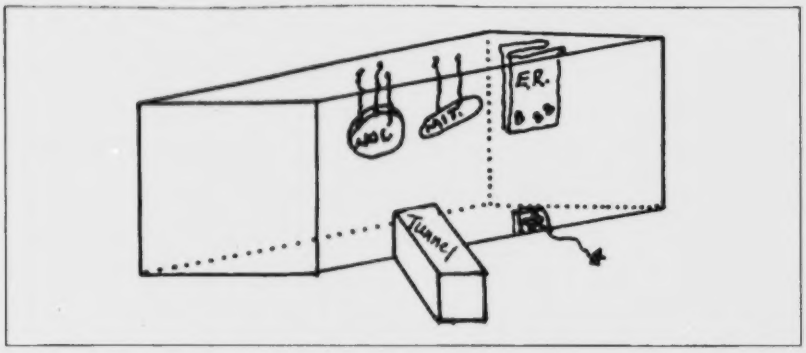

Figure 3. Sketch of tunnel taped to the outside of the cell membrane.

Figure 4. Nucleus: Note nucleolus with ribosomes forming. Note double stranded DNA. Note single stranded mRNA emerging from the nuclear pore on the left.

DNA

Have students use different colored pipe cleaners to construct the DNA double helix. Black can form the sugar phosphate sides; red, yellow, blue and green can form the nitrogen bases. Assemble 50 to 60 of them. Have the students make a few in the process of replicating.

mRNA

Use a fifth color pipe stem to represent the uracil nitrogen base. Make several single strands of mRNA. Be sure one is being formed on an open "bubble" of DNA. Two strands of mRNA should be inserted in the nuclear pores to show how they enter the cytoplasm from the interior of the nucleus.

To Assemble the Nucleus

Spray paint 3 to 4 sheets of newspaper flat black. Crumble lightly so that only the black is visible. Put this in the bottom of the nucleus and around the insides. This builds up the interior of the nucleus so it is light weight and lifts its contents to the rim. Hold the newspaper in place by spraying the interior of the nucleus with spray adhesive. Put the nucleolus on the flat black newspaper. Spray additional black newspaper with adhesive and press it around the edge of the nucleolus to anchor it in place. Glue 1 or 2 ribosomes to the surface of the nucleolus. Use hot glue.

Chloroplast

Fill a big garbage bag with packing peanuts. Cover half of it with papier-mâché and let dry. Remove the peanuts and bag. Spray paint green. Cut several round oatmeal boxes into ringed "thylakoids." Put several together and glue to the interior of the chloroplast. Use hot glue for this.

Endoplasmic Reticulum

Tape large sheets of thin transparent plastic (doubled to form 2 layers) to the ceiling of the interior of the cell and let it hang down 4 to 5 feet. Use monofilament line to tie 10 to 15 ribosomes to the endoplasmic reticulum. Glue a strand of mRNA to 1 or 2 ribosomes to show protein synthesis (see Figure 8).

Assembly

Thread monofilament line through each organelle at 2 or more points. Knot, and seal both knot and attachment points with hot glue. Put fishing hook swivels on the ends of the lines of every attachment. Make the free end of the lines about 4 inches long.

Inflate the cell, then carry organelles and a chair inside. Decide placement of each organelle, punch holes in the ceiling, and thread monofilament line through the holes. This may be easier if the cell is partly inflated so someone can walk around outside and help. For additional protection of the cell membrane you may want to put silver dollar-sized pieces of clear milk jug plastic with 2 holes punched in it on the outside of the cell at each place where the monofilament line goes through the membrane. Thread the line through the membrane, through the milk jug plastic, and back through the membrane. Knot the lines and seal the knots with hot glue. Put a loop knot in the free end of the lines and seal the tie spots with hot glue.

Use monofilament to tie the ribosomes to the endoplasmic reticulum hanging from the ceiling. Hot glue seal the knots. Hot glue several strands of mRNA to ribosomes to indicate protein synthesis.

Teaching/Learning Opportunities

The teacher presents introductory comments on the cell construction and encourages the students to suggest what items need to be constructed for the cell. Items, methods and materials are listed on the board and in the student notebook. There will be items familiar to the teacher (balloons, fishing line swivels, papier-mâché, monofilament line and garbage bags) and

Figure 5. Mitochondria: Note cristae with enzymes made from "puff paint" and swivel hooks to attach to ceiling lines.

items unfamiliar to the teacher (hot glue guns and puff paint). Puff paint is known under several brand names and is found in arts, crafts, hobby stores, and the art department in schools. For some reason it is familiar to all people aged 6 to 15, but not familiar to adults. Every item should be discussed in terms of accuracy, durability, practicality and *weight*. Only after student consensus is reached should items be put on the "official list."

Once the materials list is complete, the class should divide (with appropriate teacher input) into several groups that will work together for the duration of the project. The teacher should appoint a male and a female student to be group leaders. This position should rotate every day, or every two days. The teacher should *make every effort* to resist answering questions from this point on. All questions should be referred to the group leader. This forces a shift in the familiar paradigm of teacher/teller-student/doer to teacher/resource-student/developer. The group leader will learn that more is accomplished by asking rather than telling, and the group will learn to rely on itself to resolve problems as they develop. It is important from this point on that the teacher never have a conversation with a team member unless the group leader is present and that the teacher refer all questions to the group leader for resolution. The group leader should be encouraged to determine how the group feels a problem should be resolved. With rare exception, the teacher's response to all questions should be one of the following:

- Have you asked the group or the group leader?
- What do you think?
- How do you think that might be accomplished?

The thrust of the teacher's work at this point is to guide the group to develop confidence in itself to make decisions and to rely on the group leader for guidance. The teacher serves as a resource to the group *through the group leaders*. This is hard for teachers to do. After about three days the groups start to jell and magic happens: they become teams and work progresses in a beautiful way. At the conclusion of the project everyone has been a group leader. The teacher and the students recognize the natural leaders and those who are just beginning to develop leadership skills. Since the teacher only interacts with the group leader, this provides an opportunity to assist him or her in developing leadership skills in a day or a period in a one-on-one format. At the conclusion of the project, the class has become a team, capable and ready to tackle anything. They will be ready—and anxious—to provide "guided tours" to other classes in the school.

Conclusions

It is important to remember that students designed the organelles. Their creative constructions had a strong sense of accuracy. The construction process improves interest in learning about the cell and channels energy in a productive manner. The project's need of math for measuring is a "first" for some students; some have never taken knowledge from one class and used it in another one. They grin with understanding when they learn the definition of "transfer of learning." One student actually calculated how

Figure 6. Golgi apparatus with vesicles budding off. On right, a vacuole with compounds inside.

Figure 7. Students discussing a Golgi apparatus. The photograph also shows the nucleus, endoplasmic reticulum, ribosomes and mitochondria.

much larger the giant cell was compared to a cheek cell.

When the students realized they could take strangers on tour, their self-esteem and confidence soared, they relaxed, and they began to think as they answered questions from their tour groups. The development of self-confidence, improvement in leadership, and small group work was almost measurable from day to day. There was an enthusiastic consensus among the students that more learning and understanding occurred during the project than they thought would occur. The teacher also relaxed a lot and continues to use this method for teaching other topics.

The students compiled a complete set of drawings, calculations, sources for the plastic, and other materials as well as directions for construction of the cell and its organelles. This is available at no cost from the author. Contact: Dennis L. Stockdale, Science Department, Asheville High School, 419 McDowell St., Asheville, NC 28803.

Figure 8. Endoplasmic reticulum with two ribosomes attached. The ribosome on the right is reading a strand of mRNA.

Biochemistry of Cells

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Biochemistry is the study of the chemical constituents of cells and how they are altered during the cell's metabolic reactions. To understand how the cell works as a whole, it is important to understand each component of the cell and its function, thus comprehending its role in the biochemistry of the entire cell.

While other lab exercises allow the student to isolate and study one component of the cell, the purpose of this lab is to break down the cell into several components and perform simultaneous assays to determine the constituents. This objective is achieved through the use of differential centrifugation as a separation technique. Separation of cell components using differential centrifugation depends upon differences in size, shape and density that result in different velocities of sedimentation. Organelles with a high sedimentation rate will collect on the bottom of the tube when centrifuged at low speeds. By increasing the gravitational force (g force) and spin time used to pellet cell components, students can isolate different organelles and study them individually.

The centrifugation instructions given in this lab are for use with a standard clinical centrifuge, or an SS34 centrifuge rotor, as indicated. However, if you are not using one of these rotors, use the following formula to determine the angular velocity to use for your rotor:

$$N = 299 * \sqrt{\frac{RCF}{r}}$$

where N is the velocity in revolutions per minute (rpm), RCF is the relative centrifugal force in G's, and r is the average radius of the tube during centrifugation, that is, the distance from the center of the rotor to the middle of the tube.

In this lab, students will learn about centrifugation, determine the conditions required to separate certain organelles, and employ various biochemical and microscopic techniques to study the organelles being isolated. Several of the biochemical assays include the use of a spectrophotometer, including a quantitative assay of mitochondrial enzymes.

The organelles studied in this lab can be obtained by lysing and centrifuging plant cells. We have chosen peas as the tissue of choice for this experiment. Dried peas may be stored indefinitely and rehydrated a few hours before use; and peas contain large quantities of starch storage granules (amyloplasts), chloroplasts and mitochondria.

The first organelles that will pellet in this experiment are the amyloplasts. These membrane-bound organelles serve as starch storage compartments. Since seeds must contain large stores of energy for their initial growth, we find large quantities of amyloplasts, as one might expect. Amyloplasts are mostly starch, and thus may be identified by their intense purple staining with iodine.

The nucleus of the cell is the main location of the genetic material, DNA. Therefore, the presence of nuclei can be detected by testing for the genetic material in the sample. DNA reacts with a diphenylamine solution to generate a blue color.

Chloroplasts are plastids that contain the pigment chlorophyll and enzymes needed to carry out photosynthesis. They are the site of the conversion of sunlight to ATP, the major energy source of the organism. These organelles are easily seen microscopically as floating, green ovals.

Mitochondria, the "power-house of the cell," are the sites of aerobic respiration and most of the ATP generation. They can be detected by testing for the presence of the mitochondrial enzyme succinic semialdehyde dehydrogenase, an enzyme in the electron transport chain. This experiment measures the ability of the enzyme to transfer electrons to an artificial electron acceptor, DCPIP, which then becomes reduced and loses its blue color.

This lab also includes a protein assay to observe the relative protein content of the various organelles. Then, by calculating the mitochondrial activity per total protein content ratio, the students can determine the purification achieved by centrifugation.

Procedure

1. Solutions & Reagents

To prepare NaCl buffer solution:
0.3M NaCl
0.02M Trizma Base.
HCl as needed to bring pH to 7.5.
Keep cold.

To prepare Diphenylamine solution:
Dissolve 1.5 g diphenylamine in 100 ml glacial acetic acid.
Add 1.5 ml concentrated sulfuric acid to the solution and store in a dark bottle for up to 2 days.

To prepare protein buffer:
—First prepare phosphate buffer from 0.1M Na_2HPO_4 and 0.1M NaH_2PO_4 by mixing 3.5 parts Na_2HPO_4 with 1.0 part NaH_2PO_4, and using 1M NaOH or HCl to bring pH to 7.0.
—Then add 2.5 g serum albumin to 100 mL of the 0.1M buffer, pH 7.0.

The following materials may be purchased from Sigma Chemical Company:
Trizma Base, product #T-1503
Methylene blue, product #M-9140
Diphenylamine, product #D-8882
DCPIP, product #D-1878
Succinate, product #S-7501
Bovine Serum Albumin, product #A-6793
Total Protein Reagent, catalog #541-2
Herring Sperm DNA, product #D-7290
Lugol's Iodine solution, product #L-6146, dilute 2-fold for use

2. Preparation

Approximately 150 grams of dried peas should be soaked in cold NaCl buffer for two hours to overnight. At the beginning of the lab period, blend the peas for one minute at high speed in a Waring blender with 500 ml of the ice cold buffer. Distribute the homogenate to the students.

Each pair or group of students should filter the homogenate through 3–4 layers of cheesecloth held over a 250-ml beaker. Instruct the students to pick up the corners of cheesecloth and

make a "bag" with it, squeezing gently to remove much of the liquid. Measure and pour 30 mL of the liquid, called the filtrate, into one centrifuge tube. Keep the remainder of the filtrate on ice to be used later. (We used Oak Ridge 30 mL centrifuge tubes, #3119–0030).

The students should mark the tubes with their initials on tape, properly balance them for centrifugation, and place them into the centrifuge. Centrifugation should be done at 4° C if refrigeration is available. Keep samples on ice while waiting to centrifuge.

Centrifuge for 10 minutes at 18 g (about 400 rpm in a clinical centrifuge). Pour off the liquid, or supernatant, into another centrifuge tube, and keep the pellet on ice for biochemical studies.

Centrifuge the supernatant for 10 minutes at 175 g (1500 rpm in an SS34 rotor). Meanwhile, resuspend the first pellet with 5 ml of the NaCl buffer and keep on ice.

After the second spin is complete, pour off this supernatant into a third centrifuge tube and spin for 15 minutes at 7800 g (10,000 rpm in an SS34 rotor). Resuspend the second pellet with 5 ml of the buffer and keep on ice. After the final centrifugation is complete, pour the final supernatant into a fourth tube and keep on ice. Resuspend the third pallet with 5 ml of the buffer and place on ice.

If you are not using an SS34 rotor, use the formula described in the introduction to determine the angular velocity (in rpm) for your rotor.

3. Microscopic Assay of Organelles

This procedure allows the students to visualize the different components of the cell they are studying and identify the centrifugation conditions that isolate each component. Each pair of students should prepare two slides of each of the three pellets and the final supernatant by placing a drop of the appropriate sample on a clean slide. There should be eight slides in all. Add a drop of Lugol's iodine solution to the sample on one of each pair of slides. This will stain starch, and thus make any amyloplasts present appear as large, purple circles. On the other slide, place a drop of the methylene blue. This dye will stain the DNA of any nuclei present and make them more visible. Observe each slide under the microscope under 100X to 400X total magnification to look for the different components. The students should be able to locate amyloplasts (stained purple), nuclei (stained blue), and chloroplasts (green from their own pigment). Record the preparation in which each of the components appears.

Table 1. Starch test.

Tube #	*Pellet 1*	*Pellet 2*	*Pellet 3*	*Final Supernatant*	*Iodine*	*Color:*
1	1 ml				2 drops	
2		1 ml			2 drops	
3			1 ml		2 drops	
4				1 ml	2 drops	

4. Biochemical Assays

A. Qualitative Starch Assay: Lugol's Iodine

This procedure is a qualitative assay of each of the four samples to see if starch is present, stored in the amyloplasts. The students should obtain four test tubes, labeled for the three pellets and the final supernatant, and prepare as in Table 1.

Mix each tube until homogeneous. Record the color of each tube, noting that a purple color indicates the presence of starch in the sample.

B. Qualitative DNA Assay: Diphenylamine

This procedure tests for the presence of DNA, hence the nucleus of the cell by testing with a diphenylamine solution which turns blue in the presence of DNA. Students should obtain 6 tubes and prepare them as in Table 2.

Place a piece of parafilm over each tube and mix by gentle inversion. Place the tubes in a boiling water bath for 15 minutes. Compare the four sample tubes against the control tubes. The presence of a blue color indicates that the diphenylamine has reacted with DNA, hence there are nuclei present in the sample. Record any change in color.

C. Quantitative Assay of Mitochondria: DCPIP Reduction

This procedure tests for the presence of the mitochondrial enzyme succinic dehydrogenase. The rate of enzymatic reduction of the blue dye DCPIP is proportional to the enzyme concentration. As mitochondria carry out respiration, they transfer electrons to the dye, which becomes colorless as it is reduced. The rate of loss of color is easily measured by the use of a spectrophotometer.

Prepare 6 tubes as in Table 3.

After adding the samples to be tested for mitochondria last, mix each tube until homogeneous. Set the spectrophotometer to 600 nm, and calibrate or "zero" it. Take a reading on each tube at Time = 0 and record. Let the tubes incubate in a 37° C water bath. Take a reading on each tube every 15 minutes for 1 hour, remembering to set the spectrophotometer as before with the positive and negative controls. Record the absorbance of each tube at each interval. The rate of decrease in absorbance [calculated as OD units per time period] is proportional to the concentration of active mitochondria in each sample.

D. Quantitative Protein Assay: Total Protein Reagent

This procedure will allow the students to test the purification methods of centrifugation by testing for the concentration of protein in each sample,

Table 2. DNA test.

Tube #	*Pellet 1*	*Pellet 2*	*Pellet 3*	*Final Supernatant*	*Standard DNA [10 µg/ml]*	*Distilled Water*	*Diphenyl-amine*	*Color:*
1	0.5 ml					0.5 ml	1 ml	
2		0.5 ml				0.5 ml	1 ml	
3			0.5 ml			0.5 ml	1 ml	
4				1 ml		0.5 ml	1 ml	
5*					1 ml	0.5 ml	1 ml	
6**						1 ml	1 ml	

*Positive control
**Negative control

Table 3. Quantitative mitochondrial assay.

Tube #	*Pellet 1*	*Pellet 2*	*Pellet 3*	*Final Supernatant*	*Filtrate*	*0.1M Succinate*	*Protein buffer*	*H_2O*	*DCPIP (2×10^{-4}M)*
1	1 ml					1 ml	3 ml		1 ml
2		1 ml				1 ml	3 ml		1 ml
3			1 ml			1 ml	3 ml		1 ml
4				1 ml		1 ml	3 ml		1 ml
5					1 ml	1 ml	3 ml		1 ml
6*						1 ml	3 ml	1 ml	1 ml
7**	1 ml					1 ml	3 ml	1 ml	

*Negative control
**Positive control

from original filtrate to final supernatant. Total Protein Reagent reacts with peptide bonds of proteins and will form a purple color which has an absorbance maximum of 540 nm. The intensity of the color can be measured by a spectrophotometer and is proportional to the protein concentration in the sample. The students should obtain a set of 7 tubes and prepare them as in Table 4.

After adding the samples to be tested for protein last, mix each tube until homogeneous and incubate for 10 minutes at room temperature. Use the control blank to adjust the Spec 20 to absorbance = 0. Set the transmission scale to zero while the chamber is empty.

Read and record the absorbance of each tube at 540 nm. To compare the concentrations of protein in each sample, the students must first divide the absorbance readings recorded for the tubes containing resuspended pellets by 6. This is due to the fact the pellet samples were diluted in only 5 ml of NaCl buffer, whereas the original filtrate giving rise to the pellets was 30 ml. Thus, the pellet samples are 6-fold more concentrated than the others. After adjusting for this difference, the students can compare the results to determine what cell fraction contains the most protein.

To get a better idea of the purification obtained by this experiment, students can compare the concentration of protein [in either relative absorbance units or actual concentrations] found in the experiment above, with the mitochondria content [in enzyme activity units per unit time]. Divide the mitochondria content by the protein content to measure the relative purity of the mitochondria in each tube.

Expected Results

The amyloplasts are expected to sediment the fastest. Thus, the first pellet should be largely white [with a bit of green from contaminating chloroplasts] and should react strongly in the iodine test.

The nuclei should sediment next, followed by the chloroplasts. Of course the chloroplasts may be followed by the green color of the pellet. Both the second and third pellets will probably have large amounts of chloroplasts. The nuclei, detected using the DNA test, should be located in the second pellet.

The mitochondria are not expected to pellet with the speeds used in this experiment. Thus, the mitochondrial activity should remain in the final supernatant, as should the majority of the protein.

Table 4. Quantitative protein assay.

Tube #	*Pellet 1*	*Pellet 2*	*Pellet 3*	*Final Supernatant*	*Filtrate*	*Protein Standard (2.5 mg/ml)*	*Protein Reagent*	*H_2O*
1	60 µl						3 ml	
2		60 µl					3 ml	
3			60 µl				3 ml	
4				60 µl			3 ml	
5					60 µl		3 ml	
6*							3 ml	60 µl
7**						60 µl		3 ml

*Negative control
**Standard/Positive control

Chymosin for Classroom Experiments

An Introduction to the Nature & Action of Enzymes

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The Story of Chymosin

When Little Miss Muffet sat on her tuffet, her frugal repast of curds and whey was coagulated milk—the first intermediate in cheese-making. Her meal was probably the product of the acidity from milk souring, the result of microbial action. Addition of acid such as vinegar also causes coagulation. When the semi-liquid curd from these processes is freed from the liquid whey by filtration, it can be used as the starting point for some cheeses. However, the best-tasting cheeses are made by using the enzyme rennin extracted from calf stomachs to convert milk to curds and whey. The calf-stomach extract was used for generations in commercial cheese-making under the name rennet. Your grandmother may have purchased the dried extract at the local grocery store for preparation of a pudding-type dessert called junket.

The active ingredient in rennet extract is the enzyme rennin, now called chymosin. It has been traditionally obtained in purest form from the stomachs of unweaned (milk-fed) calves. Rennet extract can also be obtained from the stomachs of adult cows and from other milk-producing species: goats, sheep, horses, zebra, and even camels. In these cases, however, the extract usually contains other enzymes, which produce bitter off-flavors.

With the advent of genetic engineering it became possible to prepare pure chymosin without extracting calf stomachs. Molecular biologists isolated the gene for prochymosin, a protein precursor of chymosin itself, from cells in the lining of the calf stomach and inserted that gene into *E. coli* and other common microorganisms using recombinant DNA methods. The resulting "recombinant organism" was then induced to produce copious quantities of prochymosin by the process of fermentation. A description of the process for *E. coli* is given in Flamm 1991.This first novel step was followed by conversion of the prochymosin to active chymosin (Figure 1) by adjusting the pH of the protein solution to about 4.5 with hydrochloric acid, the same method used for centuries to prepare rennin from the extract of calf stomachs. The old and new methods are compared in Figure 2.

It is worth noting that the microorganisms were engineered to produce prochymosin rather than chymosin itself because the genetic engineering strategy was much simpler and more certain to produce a product identical to chymosin prepared in the traditional way.

Chymosin produced by fermentation was approved for sale by the U.S. Food and Drug Administration in 1990. This was the first genetically engineered food additive to receive FDA approval. It is now manufactured in specially designed facilities and sold throughout the United States as the premiere enzyme for cheese manufacture. Chymosin produced by fermentation is identical to chymosin produced by the stomach of the calf. Proof of this identity was the basis for FDA clearance of fermentation-produced chymosin for food use (see Flamm

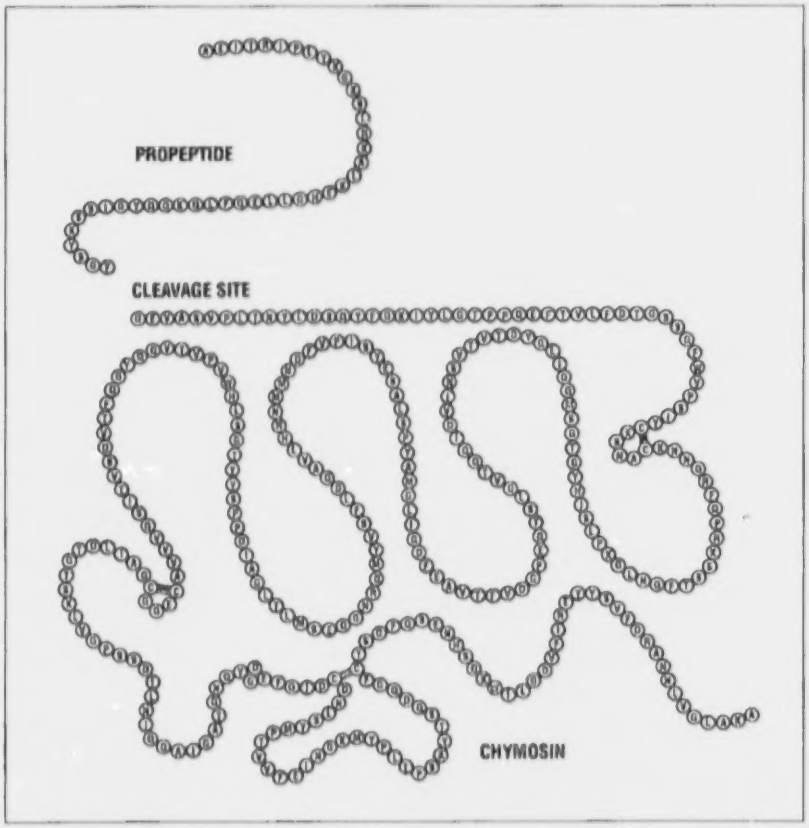

Figure 1. Chymosin and prochymosin, showing site of cleavage of prochymosin by acid.

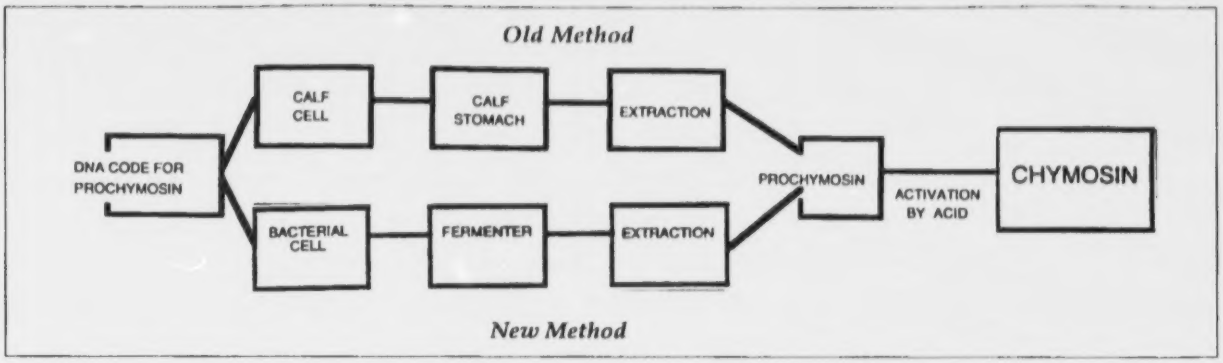

Figure 2. Chymosin manufacturing methods.

1991). The genetically engineered product has largely replaced the product extracted from calf stomachs because the former is purer, less expensive, and available in unlimited supply.

For a lively portrayal of the steps in the development of the manufacturing method for fermentation-based chymosin, see the video titled, "Biotechnology; Careers for the 21st Century," available from NABT.

How Does Chymosin Work?

The enzyme chymosin is made up of 323 amino acids (Figure 1) and has a molecular weight of 35,700. Some enzymes react with other proteins, breaking a long chain into smaller chains. These enzymes are called proteases. Chymosin is a protease that reacts with casein protein in milk.

Milk is an emulsion—a mixture of two normally immiscible liquids, fat and water, held together by a natural emulsifying agent called casein, the name for a family of proteins. If casein is destroyed, the emulsion separates into its immiscible components and insoluble calcium caseinate precipitates as the curd. Chymosin reacts almost exclusively with one key member of the casein family called *kappa* casein, which is therefore referred to as the natural *substrate* for chymosin. Made up of 169 amino acids with a molecular weight of 19,000, kappa casein is about half the size of chymosin. Chymosin cleaves the long kappa casein polymer in only one place—between amino acids 105 and 106 in the chain, forming two large protein pieces. Other proteases can react with casein and coagulate milk, but since they are less selective than chymosin, they create more fragments, some of which produce off-flavors in cheese. The most common of these proteases is the enzyme pepsin, often found with chymosin in the stomachs of adult animals. For a survey of the many factors involved in milk clotting, see Berridge 1954.

Classroom Use

The protease chymosin is particularly well suited for classroom use at all grade levels because it is safe, the endpoint—curd formation—can be observed with the unaided eye, and experiments can be performed rapidly with simple equipment. Previously described experiments (Gill 1987; Wheelock 1971) used rennet (calf stomach extract) or rennilase (a microbial protease) as the enzyme source with the problem of considerable batch-to-batch variation in activity. Genetically engineered chymosin is purer (clear liquid vs. red-brown solution) and gives more reproducible results. Earlier reports used complete clotting of milk as an endpoint, which gives highly variable results. The endpoint measurement described below is more reproducible. Finally, the use of genetically engineered chymosin broadens the teaching arena considerably, providing a basis for classroom discussion of recent technological advances that are interesting to teachers and students, such as the sequencing of the human genome, gene therapy, the cloning of mammals, and the production of genetically improved foods.

Many of the experiments outlined here can be carried out at lower grade levels. Others, such as serial dilution, can be reserved for use in more advanced classes.

Objectives

The goal of these experiments is to familiarize the student with some of the fundamental aspects of enzyme/substrate interaction. Successful completion of these assignments will provide valuable experience with the properties of enzymes, including the effect of concentration, temperature and pH on their activity; denaturation of enzymes; and the nature and properties of proteases.

Materials & Methods

- Chymax® (chymosin manufactured by fermentation[1]
- Milk—various fat levels
- Water (tap water can usually be used)
- White vinegar (5% acetic acid)
- 5% sodium bicarbonate solution
- 500 ml beakers
- 50 ml beakers
- Plastic droppers
- Pipets, graduated in 0.5 and 1.0 ml volumes
- Water bath
- Test tubes of 20 ml capacity
- Universal pH paper
- Thermometer
- Ice
- Timer

General Purpose Protocol

To ensure a coagulation time of about 10 minutes, which is practical for classroom use, aging milk in advance at room temperature for 6 to 8 hours is helpful when working at 20 to 25° C. Microbial action lowers the pH, facilitating coagulation. As an alternative to aging, one can add two drops of white vinegar diluted 1:4 (1% acetic acid) to 5 to 10 ml of milk. However, for higher temperatures (30

[1]The commercial product, CHY-MAX, is sold by Chris Hansen and Co., 222 State Street, Milwaukee, WI. The liquid product contains approximately 0.1% active chymosin. It should be stored in a refrigerator to minimize loss of activity.

to 37° C), aged milk is not necessary, as the reaction is very rapid.

Endpoint Observation

The endpoint should be the first sight of a fine precipitate. Since this is a white precipitate against a white background, some practice is required to achieve reproducible results. Best results are obtained by tilting a test tube on its side and turning between the fingers. Viewing the test tube in a bright light is helpful. The first sign of coagulation is a thickening (creaming) in the film along the side of the test tube. Particulate matter is observed soon thereafter. Once curdling begins, the process is essentially exponential and complete clotting occurs rapidly. This process is especially rapid at higher temperatures (30° to 37°) where substantially decreased concentrations must be used for a 5 to 10 minute endpoint.

Complete clotting can also be used as the endpoint (Gill 1987; Wheelock 1971), but the results tend to be more difficult to reproduce. Complete clotting will finally occur in all the experiments described here, as the change from fine precipitate to clot is rapid. Observation of clotting may enhance student interest as the first step in making cheese.

Effect of Enzyme Concentration on Chymosin Activity

Preparing a Standard Enzyme Preparation (Stock Solution)

Commercial chymosin solution can be used as received as the stock solution, but at this concentration chymosin can coagulate milk very quickly at temperatures above 30° C. If necessary, it should be diluted until the average time for milk clotting is about 10 minutes.

Two approaches for determining a useful working concentration are described below. Both enable the experimenter to also determine the effect of enzyme concentration on the speed of reaction.

Student Activities

Qualitative Approach

Label five test tubes 1 through 5. Add 5 ml of regular, aged homogenized milk to each. Adjust the temperature to 25 to 30° using a water bath. Add 2 drops of commercial enzyme solution to the first tube of milk, 4 to the second, 6 to the third, and 8 to the fourth. Do not put any enzyme in the fifth tube. Record the time for the milk in each tube to begin to coagulate. Prepare a table similar to Table 1 to record your observations. For further experiments, use the concentration of chymosin that begins to clot milk in about 10 minutes.

Table 1. Record of coagulation time.

TEST TUBE NO.	DROPS OF ENZYME	COAGULATION TIME
1	2	
2	4	
3	6	
4	8	
5	0	

Prepare a graph of the data like Figure 3.

Question

The fifth tube, containing no enzyme, is called a negative control. Why do we include this in the experiment?

Quantitative Approach—Serial Dilution

In this case the dilution at each step is 10-fold.

1. Place seven test tubes in a test-tube rack; number them 1 through 7.
2. Pipet 2.0 ml of distilled water into each tube.
3. Add 0.5 ml of commercial enzyme to tube #1 and mix gently.
4. Transfer 0.5 ml of the mixture from Tube #1 to Tube #2 and mix.
5. Repeat the transfer process until Tube #7 is reached.
6. Discard the 0.5 ml that is removed from Tube #7.
7. You should now have 2.0 ml in each of the seven tubes.
8. Quickly add 8 ml of aged milk to each tube, mix gently.
9. Let stand at room temperature (22° C) for 10 minutes.
10. At the end of 10 minutes, observe each tube for clotting and record your results in Table 2. Alternatively, observe and record the time for the curdling process to reach the endpoint, as described above.
11. Let all tubes stand overnight at room temperature. What do you observe?

Prepare about 20 ml of the concentration of chymosin that clots milk in about 10 minutes. This is called the stock solution or standard enzyme preparation. Reserve this for further experiments outlined below.

Conclusions

1. What conclusion can you draw about the effect of enzyme concentration on the speed of reaction with a fixed volume of milk?
2. What is the concentration of chymosin in Tube #7?

Students should be able to calculate the chymosin concentration in each tube since each contains one-tenth as much enzyme as the previous one. Commercial chymosin is about 0.1% active enzyme.

Temperature Effects on Chymosin Activity

Enzyme activity is strongly influenced by temperature. Each enzyme exhibits maximum reaction rate at a certain temperature. (Most of the enzymes in the human body work best at body temperature [37° C]). However, if the temperature is raised high enough, the enzyme becomes denatured and loses all activity. In this set of experiments these effects are investigated.

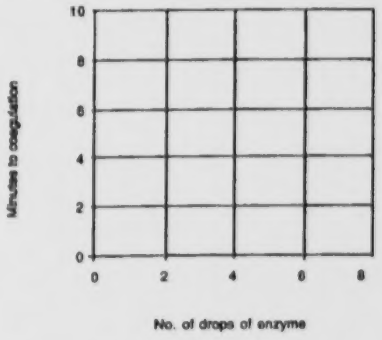

Figure 3. Sample graph.

Table 2. Record of enzyme concentration and related observations.

TUBE	ENZYME CONCENTRATION	OBSERVATION
1		
2		
3		
4		
5		
6		
7		

Student Activities

Rate of Reaction

1. Pipet 8 ml of whole milk into three test tubes labeled 1 through 3. Hold Tube #1 on ice (4° C), Tube #2 at room temp (22° C), and Tube #3 at 37° C (use a water bath or just hold the tube under your armpit).
2. Allow each tube to adjust to the surrounding temperature (approximately 5 minutes).
3. Quickly add 0.5 ml of the standard enzyme preparation to each tube.
4. Return the tube to the appropriate temperature and incubate until the milk clots.
5. Place all tubes into boiling water after clotting is observed. Record results in Table 3.

Questions

1. How does heat affect the clotting time of chymosin?
2. How do you explain the results observed in Tube #1?
3. Why are the tubes placed in boiling water after clotting?

Enzyme Denaturation

If a solution of chymosin is maintained at about 60° C, it will rapidly lose activity. For this experiment, first make one measurement with chymosin and milk at room temperature, selecting the number of drops of chymosin that will clot milk in 5 minutes or less. This short time is necessary for practical classroom use as the heated samples will take longer to react.

Maintain about 2 ml of chymosin at about 60° C in a water bath. Now set up six test tubes at room temperature, each containing the same amount of milk as the previous experiment. Every 5 minutes, add to one of the tubes the same number of drops of 60° C chymosin solution as used for the room temperature experiment. Observe the time for clotting to begin. The results, when plotted, will produce a graph like Figure 4.

The data can also be converted into a form that enables construction of a graph of time to clot vs. percent residual enzyme activity.

The Effect of pH on Chymosin Activity

The activity (rate at which reaction takes place) of enzymes varies with pH. Each enzyme has its greatest activity at a certain pH, called the pH optimum. This set of experiments is designed to investigate the change of activity of chymosin with pH. Note that too much acid will curdle milk even in the absence of chymosin. Even white vinegar (5% acetic acid) can cause some curdling. Therefore, we recommend that vinegar be diluted 1:4 with water. About 5 drops of this concentration in 5 ml of milk produces a pH of about 6.5.

Student Activities

1. Add 10 ml of milk to each of three small beakers or test tubes of 20 ml capacity.
2. Add 1% acetic acid (white vinegar diluted 1:4) dropwise to the first beaker until the pH is adjusted to 5.0.

Table 3. Record of temperature, clotting time and observations.

TUBE	TEMP	CLOTTING TIME	OBSERVATION
1			
2			
3			

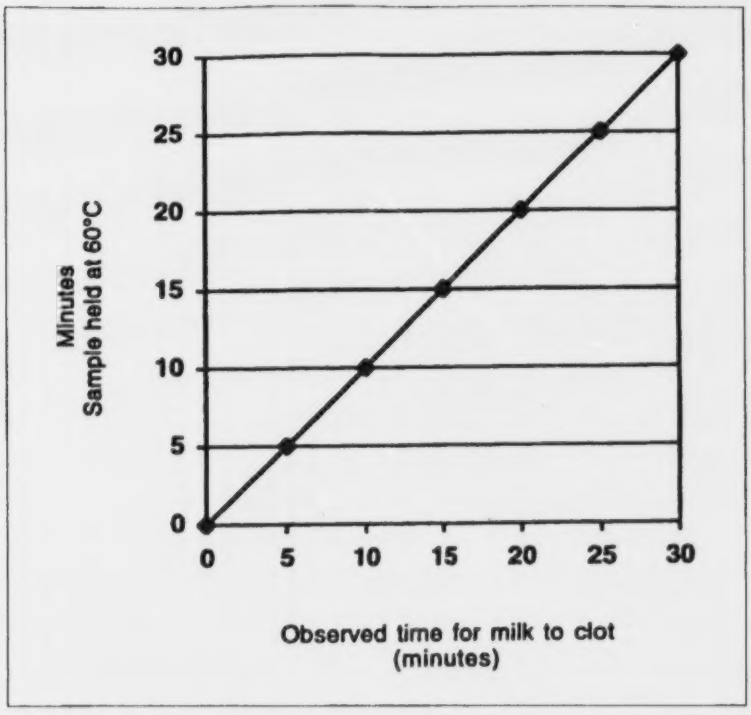

Figure 4. Sample graph.

3. Add a 5% solution of sodium bicarbonate dropwise to the milk *in the* second beaker until the pH reaches 8.0.
4. Measure the pH of the milk in the third beaker.
5. Transfer 8 ml of milk *from* each beaker to a clean test tube.
6. Add 2 ml of the standard enzyme preparation to each tube and mix gently.
7. Incubate the tubes at room temperature and record the clotting time as described under Endpoint Observation.

Make a table like Table 3, substituting pH for temperature.

Questions

1. What is the pH of natural whole milk?
2. Explain the effects of pH on chymosin activity.
3. What pH yields the highest enzyme activity? Explain.

Explain in simple terms how temperature, pH and enzyme concentration affect the activity of chymosin with its natural substrate. Do you think all enzymes would respond like chymosin?

Integration of Math & Science

The time course of enzymatic processes lends itself very well to integrated exercises in math and science. The graphing exercises outlined above deal with one variable, e.g. time vs. temperature. For more advanced students, two variables can be considered such as enzyme concentration and temperature, e.g. "How can a dilute solution of enzyme be made to react as rapidly as a more concentrated one?" or "How can a cold solution of chymosin and milk be made to react as fast as a warm one?" Graphing exercises are appropriate here.

Additional Experiments for Students

Compare the curdling times for different types of milk: skim, 2% fat free, "half-and-half," and powdered milk. Develop a hypothesis to explain the differences. Be sure to keep all other variables constant. Why is this important?

Since milk contains calcium and the curd is primarily calcium caseinate, predict the effect of added calcium salts on the rate of reaction. Devise an experiment to test your hypothesis.

Additional Areas for Study

Proteases

Proteases have become a popular subject in the media because protease inhibitors are among the most important drugs in the fight against AIDS. In fact, proteases are important to the survival of a wide variety of disease-producing organisms, from malaria to the common cold. The search for drugs to interfere with the action of proteases is an active area of research (see Seife 1997).

Pro-enzymes

As described above, the actual product secreted by the cells in the calf stomach is prochymosin, which has no inherent enzymatic activity. It is called a zymogen.

Pepsin, also found in the calf stomach (and, incidentally, the active ingredient in meat tenderizers), is also produced as a zymogen, called pepsinogen. There are many examples of precursor proteins in nature. Insulin for the treatment of diabetes is produced initially by cells of the pancreas as pro-insulin. Cleavage of the peptide segment of pro-insulin releases insulin. Other examples of pro-proteins can be found in the literature (and on the Web).

Genetic Engineering

The use of the product of genetic engineering in these experiments affords the opportunity to discuss and investigate the subject more broadly; both technical aspects, such as gene splicing, and methods of moving genes from one cell to another; and ethical considerations.

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Glossary

Polymer — A long molecule arranged as a chain of many subunits. mer = unit poly = many
Example: A–B–A–B–A–B–A–B–

Protein — A polymer in which the subunits are amino acids.

DNA — The polymer of deoxyribonucleic acid, found in every living cell, which tells the cell to produce proteins. It is a chain of alternating sugar (deoxyribose) and phosphoric acid groups with a segment called a base attached to each sugar unit:

```
–S–P–S–P–S– – – – – –
  |   |   |
  B   B   B
```

where S = sugar, P = phosphoric acid, and B = nucleotide base. When DNA exists as a double helix, two chains are wrapped around each other in reverse fashion with one base from each chain paired with one from the other chain, hence the term "base pairs."

Denaturation — Drastic change in a protein's environment will result in a change in its 3-dimensional configuration and loss of activity. For example, heating an egg causes the clear portion to turn white. It has coagulated, lost its original 3-dimensional structure, and its original properties, including color and solubility.

Teaching Protein Synthesis Using a Simulation

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Protein synthesis is a concept that many high school students have difficulty understanding. Comprehending the process involved in making a protein is somewhat complex for an average student, and textbook descriptions and illustrations often fail to present an adequate explanation.

The purpose of this article is to describe a simulation that can be used in the classroom to clarify this seemingly formidable topic. The majority of students have commented that the activity is a lot of fun and that it has helped them understand the steps involved in protein synthesis.

It should be noted that preparing the materials for this activity is rather tedious. The materials are inexpensive, however, and once made, the activity can be repeated over and over with a minimum of preparation. The initial time invested is well worth it.

The simulation requires participation of a minimum of 21 students. If your class size is less than this, students may double up on the roles involved. The students will demonstrate how human gastric juice peptide, which is found in normal human gastric juice, is made.

Human gastric juice peptide was selected because it is composed of only 10 amino acids and its construction, therefore, would be manageable to demonstrate in the classroom. The amino acid sequence of human gastric juice peptide, according to Croft (1980), is as follows: LEUCINE ALANINE ALANINE GLYCINE LYSINE VALINE GLUTAMIC ACID ASPARTIC ACID SERINE ASPARTIC ACID

Materials Needed

Eleven 4 × 6 index cards will be needed to represent the 10 DNA codes plus one stop triplet. Three capital letters should be placed on each card (as shown in Figure 1) to represent the nucleotides found in the portion of the DNA molecule directing the synthesis of human gastric juice peptide. The letter "A" represents adenine, the letter "T" represents thymine, the letter "G" represents guanine, and the letter "C" represents cytosine.

A notch, used to visually characterize each of the four types of nucleotides in this model, should be cut into the card *beneath* each letter to represent how complementary bases "match" each other during the process of synthesis. Using the analogy of puzzle pieces, the molecular shape of an adenine nucleotide "fits" the shape of a thymine nucleotide. A guanine nucleotide "fits" the shape of a cytosine nucleotide. An RNA uracil nucleotide will "fit" the shape of a DNA adenine nucleotide.

It is helpful to number the cards sequentially on the back so that they can easily be kept in order when the activity is over. The 11 cards should look like those in Figure 1.

An additional set of 11 (4 × 6) index cards will be needed to represent the 10 messenger RNA (mRNA) codons plus one stop codon (see Figure 2.) Three capital letters should be placed on each card to represent the nucleotides found in the complementary mRNA molecule. The letter "A" represents adenine, "U" represents uracil, "G" represents guanine, and "C" represents cytosine. A characteristic notch representative of each nucleotide should be cut into these index cards *above* each letter. Attach a safety pin to each of these index cards. The 11 index cards should look like those in Figure 2.

Ten wire clothes hangers and 10 (4 × 6) index cards will be needed to represent the transfer RNA (tRNA) molecules. Twist the hangers into the shape of a cloverleaf. On the bottom of each hanger, create an anticodon site by attaching an index card with the letters representing the appropriate exposed bases. As in mRNA, the letter "A" represents adenine, "U" represents uracil, "G" represents guanine, and "C" represents cytosine. A characteristic notch representative of each base is cut into the portion of the index card *below* each of the letters. On the back side of the card, print the name of the appropriate amino acid that will attach to the amino acid attachment site of the tRNA model. The index cards for the 10 models of the tRNA molecules should look like those in Figure 3.

Finally, obtain 10 wooden balls and nine springs from a chemistry ball and stick model set. The 10 balls should contain at least two holes. Tape a small label on each identifying what amino acid the wooden ball represents. Plastic interlocking toy beads found in toy stores can be used as a nice alternative to the chemistry ball and stick model set.

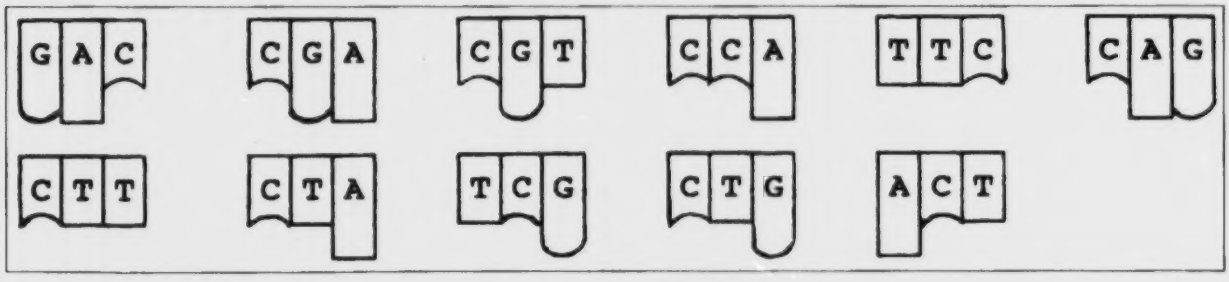

Figure 1. Index cards, shown in order from left to right, representing the DNA triplets.

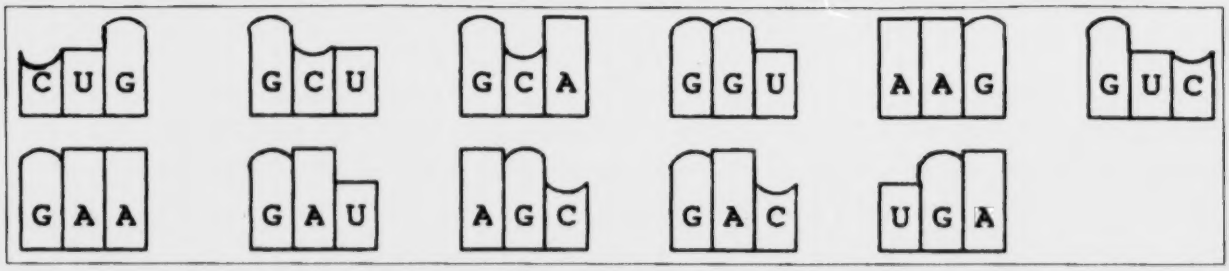

Figure 2. Index cards, shown in order from left to right, representing the mRNA codons.

For simplicity, the initiation codon is not included in this activity. If a teacher wishes to incorporate the start codon, the following additional materials must be acquired: a DNA index card bearing "T A C"; an mRNA card bearing "A U G"; a tRNA wire model with the anticodon "U A C"; a spring; and a wooden ball labeled "METHIONINE."

Procedure

Inform the students that a peptide called human gastric juice peptide needs to be constructed by a cell. Designate an area of the classroom as the nucleus of the cell and another area as the ribosome. The back lab table could represent the nucleus and the ribosome could be represented by two chairs arranged in the front of the room to form a narrow passageway. The process of transcription begins when the imaginary DNA molecule unwinds and a strand of DNA is exposed. Lay the DNA index cards (as shown in Figure 1) next to each other in the appropriate sequence on the lab table.

Next, distribute 11 mRNA codon index cards to 11 different students in the class. Instruct them to form a complementary strand of mRNA by finding their appropriate place along the exposed strand of "DNA." The students should match their codon cards to the appropriate base sequence, remain in place, and pin their cards to their shirts. A model of an mRNA molecule is now formed. The students, wearing their pinned codons, march hand-in-hand (chemically bonded now as "mRNA") out of the "nucleus" to the "ribosome" at the opposite end of the room. Be sure that the student bearing the codon "C U G" is in the lead.

Translation is the next stage in the synthesis of the peptide to be demonstrated. Give 10 different students one of the 10 cloverleaf-shaped clothes hangers. They will represent tRNA molecules. The 10 labeled wooden balls are given to the remaining students who have not yet participated. If no students are left to participate, the wooden balls can be set on a desk. Ask the "tRNA molecules" to locate their appropriate "amino acid" by simply matching the name of the amino acid on the back of their index card with the label on the wooden ball. Then have the students place the ball at the top of their hanger.

As the model mRNA encounters the "ribosome," the awaiting "tRNA molecules" (students with the clover-shaped hangers bearing the "amino acids") must recognize their appropriate codons before entering. The model amino acids are delivered one-by-one as the "ribosome" moves along the model strand of mRNA. If a complementary match is made between the codon of the mRNA and the anticodon site of the tRNA, the amino acid is released from the tRNA molecule at the ribosome. A peptide bond forms between the amino acids after each is brought to the ribosome. Springs from the chemistry ball and stick model kit will represent these bonds and should be attached as soon as the correct amino acid is carried to the "ribosome." The teacher can pretend to act as the enzyme that allows for chemical bonding to occur between amino acids. Eventually, the stop codon is reached signifying the end of protein synthesis.

This activity can be varied depending on your class size, student ability, and time constraints. The activity, as described above, will require approximately 25 minutes. Allow more time if your students are having difficulty with protein synthesis or if you want to have a discussion period following the activity.

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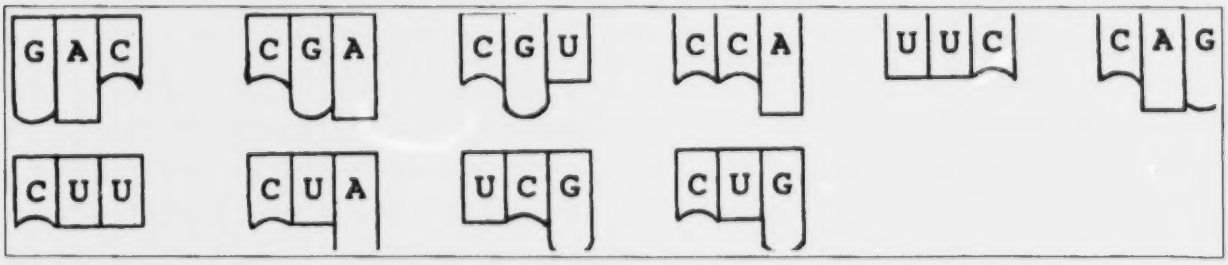

Figure 3. Index cards, shown in order from left to right, representing the anticodons of the tRNA molecules.

Microbes & Fungi

Kitchen Microbiology

(It's Easier Than You Think!)

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We are reminded continually of the presence of microorganisms in our surroundings. News reports of tainted food due to microbial contamination of chicken, beef or fruit are prevalent. Strep throat, acne and various other bacterial infections are common occurrences that are familiar to our students. As consumers, we are enticed to buy facial scrubs, mouthwashes, soaps and cleaners that inhibit bacterial growth. How effective are these products in controlling bacteria? Where do bacteria normally live? What conditions do bacteria require to live and reproduce?

These questions can be readily explored in middle and secondary classrooms using the following procedure to prepare culture media that can be used for the isolation and propagation of microorganisms. This procedure was devised and tested with the following goals:

1. To use supplies available locally in a supermarket or health food store
2. To reduce the process to its essential components
3. To produce a medium that would support bacterial growth equal to that of a professionally prepared medium.

The technique described herein is a simple and effective means to prepare the culture media needed to conduct microbiological investigations.

Table 1. Materials needed for *Kitchen Microbiology*.

- cotton swabs
- paper towels
- agar, 1 ounce, purchased at a local health food store
- measuring spoons—teaspoon and ½ teaspoon
- canned chicken broth—fat free, low in sodium, 14.5 ounces (410 ml)
- 40 sterile, plastic, disposable petri dishes, 15 × 100 mm
- 4 canning jars able to hold 8 fluid ounces
- manual can opener
- commercial disinfectant such as Lysol®
- big pot with lid—8 quart or larger
- aluminum foil
- 2 cups (500 ml) filtered or distilled water
- 1 cup (250 ml) glass measuring cup
- metal teaspoon or soup spoon
- permanent marker
- oven mitt
- tongs

Materials

All the needed materials are shown in Table 1 and can be found in a kitchen or purchased locally, with the exception of the petri dishes. The sterile, plastic, disposable petri dishes are not expensive and can be purchased from most scientific supply companies for about $4 or $5 per 20 plates. Although alternative containers have been used (Blair & Bowen 1996), there is no substitute for microbiological petri dishes, and students might feel more like they are doing "real" science when they are using common scientific equipment.

Procedure

1. Clean the kitchen counter with disinfectant and a paper towel. Do not use sponges as they are usually heavily contaminated with bacteria.
2. Wash the canning jars and measuring cup in hot, soapy water. Thoroughly rinse out all the soapy water. Turn the jars and measuring cup upside down on a paper towel to drain.
3. Place the can opener and spoon in the large pot and cover with water. Boil them for 10 minutes. Remove them from the boiling water with tongs and place them onto the paper towel next to the canning jars and measuring cup. The tongs will not be sterile, so they should be used at the *end* of the can opener and spoon, away from the end that will come into contact with the media. To determine the amount of water needed for boiling the canning jars, place the four canning jars into the pot and add water so that the level comes up about half way on the jars. Use care as the jars are empty and will tend to float. Remove the jars, replace the lid on the pot, and bring the water to a boil. While the water is being brought to a boil, prepare the agar.
4. Wash the top of the chicken broth can with hot, soapy water and rinse.
5. Add 200 ml (7/8 cup) of filtered or distilled water to each of two canning jars (use the measuring cup to measure). Then open the chicken broth and fill the remaining two canning jars with 200 ml (7/8 cup) of chicken broth. One can of chicken broth is enough for two jars. There should be two jars with chicken broth and two with filtered water. Be sure to use jars large enough so that the agar fills the jar halfway or less.
6. Add 1.5 tsp (about 3 grams) of agar to the liquid in each jar. Mix the agar with the clean spoon,

starting with the water agar first, then the chicken broth agar. This sequence prevents the transfer of protein from the chicken broth to the water agar.

7. Make an aluminum foil cover (use 2 to 3 layers for each jar). Label the aluminum foil covers with a permanent marking pen to indicate the contents of each jar (e.g. WA = water agar; KM = kitchen medium). Place each jar into the pot of boiling water. Make sure to wear the oven mitt to protect your hand and check that the water level is *at or above* the level of the liquid in the jars. Add more water if needed. Put the lid on the pot.
8. Boil the agar for 35 minutes.* Do not start timing until the water in the pot is actually boiling. Gently swirl the agar in the jars every 10 to 15 minutes. Wear the oven mitt to protect your hand. Check the water level every time you swirl and add more water as necessary.
9. While the agar is boiling, remove the sterile petri dishes from their protective plastic sleeves by cutting off the very top of the sleeve with a pair of scissors. Save the sleeve. Label 20 dishes as "WA" and 20 dishes as "KM" with the permanent marker.
10. When the boiling has finished, turn off the power source and carefully move the pot to a cool surface. Leave the canning jars in the pot while the agar is cooling.
11. When the agar has cooled (50 to 55° C or about 20 to 25 minutes), remove one jar at a time from the pot. Wipe off the water on the outside of the jar with a paper towel. Remove the aluminum foil lid and pour enough agar into the bottom of a petri dish to just cover the bottom (about 20 ml). Replace the lid and carefully slide the petri dish to the back of the counter out of the way. Make sure not to slosh the media onto the underside of the lid when you slide the plates to the back of the counter. Work steadily as the agar in the jars remaining in the pot will begin to solidify at about 45° C. Continue in this fashion until all the agar has been dispensed.

The water agar was included to demonstrate that a nutrient source is necessary for microbial growth. Steps 5, 6, 7 and 9 can easily be amended so that only the kitchen medium is prepared.

*NOTE: This procedure was developed at an altitude of 7000 feet. The time for boiling was increased by 10 minutes at this altitude.

Inoculation, Incubation & Precautions

In addition to the chicken broth agar and the water agar that were made in the kitchen, agar that was professionally prepared (Difco Nutrient Agar®) was also used for a comparison study. To maximize viewing of colonies, all agar plates were labeled on their edges with type of agar, date and substance tested. On the back of petri dishes containing kitchen media, a line was drawn down the middle of the plate and the left side labeled "C" for control. Ten different sources were tested for microbes (see Table 2). Each cotton swab was carefully removed from the box and first streaked onto the control side of the kitchen medium to determine if any bacteria were present on the swab prior to inoculation. This process also "wets" the swab, which is advantageous as moist swabs will pick up more inoculum from dry surfaces than unmoistened swabs. Alternatively, swabs can be moistened using the water droplets that have condensed on the underside of the petri dish lids. The same cotton swab was then inoculated with the test substance, streaked onto the water agar, the other half of the kitchen medium, and the professional medium, in that order. After the plates were inoculated, they were taped shut (see below) and put back into the plastic sleeves. The opening at the top of the sleeve was folded over and closed with tape. If the plastic sleeve is unusable, a large plastic bag with a sealable closure will also work.

In this study, the plates were incubated at 98.6° F (37° C) for 24 hours in an incubator. However, incubation for 3 to 4 days at room temperature works equally well, although growth of the bacteria is slower. Incubation times can be reduced by placing the plates in an area that is above ambient temperature, such as on top of a refrigerator, toward the back.

It is essential that students tape their petri dishes shut because it is possible that students will isolate pathogens and/or organisms that form spores. It is not safe for students and staff to be exposed to either of these.

Once the exercise has been completed, the cultures on the plates must be sterilized and made harmless. Unsterilized plates must not be disposed of in the garbage! The plates can be incinerated if the school district has an incinerator. Alternatively, arrangements can often be made with a local hospital, university, college or state health department to use its autoclave to sterilize the plates. If none of these alternatives is available, the plates can be sterilized in a pressure cooker for 20 minutes at 121° C and 15 pounds of pressure, and then disposed of in the garbage.

As a final word of caution, microwave ovens should never be used to sterilize the plates because:

1. Conditions for rendering the plates sterile must be arrived at empirically for each microwave.
2. Microwave ovens are almost always used to prepare food and are rarely dedicated solely to sterilization.

Results

Amount of colony growth and number of different types of colony morphologies can be easily observed and recorded. However, identifying bacterial or fungal organisms based solely on colony morphology is rarely definitive, although colonies with extensive hyphal growth (fuzzy in appearance) are usually fungal. Outdoor sources will probably produce more fungal growth than indoor sources. The vast majority of colonies recovered in this study were bacterial (Table 2).

The kitchen medium was as efficient as the professionally made medium in supporting colony growth. Very few colonies appeared on the control side of the kitchen media plates, demonstrating that either cotton swabs are relatively free of bacteria or that where colonies did appear, aseptic technique was comprised. Except for the fish tank water, almost nothing grew on the water agar plates; they contained no nutrients, demonstrating that agar is nonnutritive for most microbes. The small colonies produced on the water agar from the fish tank inoculum were a result of nutrients in the fish tank water.

To determine if this procedure was accessible to a nonmicrobiologist, an undergraduate biology education major with little experience or knowledge of microbiology volunteered to repeat the experiment. With no help or advice from the authors, the student successfully replicated the above procedure. This success has encouraged

Table 2. Comparison of kitchen medium to professional medium in growth of microbial colonies from various sources.

			Kitchen Medium		*Professional Medium*	
Test Substance	*Cotton Swab*[a]	*Water Agar (+/−)*[b]	*Growth (+/−)*	*Types of Colonies*[c]	*Growth (+/−)*	*Types of Colonies*
Banana (surface)	0	−	+	4	+	6
Bottom of Shoe	0	+ (5 colonies)	+ + +	8	+ + +	10
Ear Wax	0	−	+	3	+	4
Fish Tank Water	2	+ + (very small)	+ + +	7	+ + +	10
Kitchen Sponge	0	−	+ + +	3	+ + + +	2
Money (dollar bill)	7	−	ND[d]	ND	+	3
Plant Leaf	1	−	+	2	+	2
Stove Top	2	−	+	2	+	2
Teeth	1	−	+ +	2	+	4
TV Dust	0	+ (3 colonies)	+ +	4	+ +	4

[a]This is a control where the cotton swab was rubbed onto the agar surface before it was rubbed onto the test substances (number of colonies reported).
[b]Growth: (−) no growth; (+ to + + + +) = different amounts of growth from several distinct and well separated colonies to confluent growth, sequentially.
[c]Indicates number of different types of colony morphologies.
[d]ND: Not determined.

him to incorporate *Kitchen Microbiology* into his student teaching experience.

Experimental Issues

We have found that there is simply no substitute for two of the materials used in our study—agar and petri dishes. Fortunately, agar is readily available at most health food stores (often located in the bulk foods section as "agar-agar"). Since agar is typically available in bulk, one can weigh out as little or as much as one requires. Its lack of accessibility as a nutrient to all but a few microorganisms, its superior gelling properties, and its local availability are all advantages that agar has over cornstarch and gelatin, which have been used as gelling agents (Blair & Bowen 1996). As mentioned earlier, sterile, plastic petri dishes can be obtained from many scientific supply houses or might be available as a donation from a local college, university, hospital or clinical laboratory. Alternatively, glass petri dishes can be purchased and reused, but are far less convenient when it comes to cleaning and sterilization.

We used low sodium chicken broth directly from the can. Canned chicken broth is sterile due to the processing procedure and is much more convenient than recovering the broth from a home cooked beef roast, as suggested by Blair and Bowen (1996). They prepared homemade beef broth because it lacked any monosodium glutamate (MSG), which they considered an inhibitor of microbial growth. However, MSG serves mainly as a "flavor enhancer" and only marginally as a food preservative (Atlas 1997). Blair and Bowen also suggest adding table sugar (sucrose); however, dextrose (D-glucose, also available at health food stores) would be utilized by a larger number of microbes.

Initially we had intended to use a pressure cooker in order to reach sterilizing conditions of 15 psi at 121° C for 15 minutes, but we really wanted to see what could be done without employing any sterilizing conditions. Many items (e.g. paper towels, cotton swabs, aluminum foil) are essentially rendered sterile during production. Using previously unopened packages of paper towels and cotton swabs will help to minimize contamination. Since the chicken broth was already sterile, we only needed to wash the top of the can and boil the can opener and the spoon. The canning jars and measuring cups were washed in hot water and detergent. No other precautions were employed. The agar was weighed out from a common bottle into a plastic bag supplied by the store, and a large pot was used to boil the agar instead of using a pressure cooker.

Variations on a Theme

The protocol outlined above can be modified to use nutrient sources other than chicken broth. Examples of such alternatives and the composition of relevant nutrients in each are listed in Table 3. An excellent resource for preparing alternative microbiological media is the *Handbook of Microbiological Media* (Atlas 1993) in which recipes for Skim Milk Agar (p. 810), Tomato Juice Agar (pp. 916–17), and V-8™ Agar (p. 973) can be found. There are a few technical issues to note when preparing media from the nutrients listed in Table 3. Skim milk powder contains spores (just as regular, pasteurized milk does) which are difficult to kill without autoclaving or using a pressure cooker. We have found that Pet™ and Carnation™ brands of skim milk powder have fewer spores than other brands. Spores in the skim milk that are not killed during preparation will germinate upon incubation and will form lenticular ("lens-shaped" or "football-shaped") colonies inside the agar. The skim milk powder should

Table 3. Alternative nutrients for microbiological media.

Nutrient	*Protein (g/200 ml)*	*Sugars (g/200 ml)*
Skim Milk[a]	6.7	10.0
Chicken Broth	2.5	0.83
Beef Broth	1.7	0.83
Vegetable Broth	0	2.5
Carrot Juice	1.1	8.0
Tomato Juice	1.2	5.3
V-8™ Juice	1.2–1.7	6.5
Orange Juice	0.83	23.3

[a]Reconstituted to one cup (240 ml)

be heated gently (to avoid coagulation) and then brought to a boil so that it is fully dissolved. Note that skim milk agar can be prepared using just skim milk powder, agar and water (Atlas 1993); or, the skim milk powder can be added directly to the chicken broth agar (1.5 g powder/200 ml of water or chicken broth). If skim milk agar is successfully prepared, it will be white and opaque. It is a wonderful medium as many of the organisms in the environment secrete proteases (enzymes that break down protein) and zones of cleaning will appear around these colonies as the milk protein is broken down.

Fruit juices generally contain insufficient protein (Table 3) to be used alone and should be mixed with chicken broth agar. However, fruit juices can be purchased as concentrates and are rich in sugars. We recommend mixing fruit juice concentrates with chicken broth in a ratio of 1:4 (e.g. 40 ml fruit juice concentrate to 160 ml chicken broth). This combination will make a rich and colorful agar medium. Similarly, tomato juice and carrot juice can be mixed with chicken broth in a ratio of 1:2 (e.g. 67 ml of tomato juice to 134 ml chicken broth). V-8™ agar can be made with just V-8™ juice and agar (Atlas 1993) if the variety of juice contains about 2 g of protein (alternatively, it can be mixed with chicken broth as described for tomato juice). Finally, one should check the pH of the medium when it contains fruit or vegetable juice. These additives will often lower the pH between 4 and 6, which will prevent many organisms from growing. The pH can be adjusted to neutrality by adding a little base or, two sets of agar plates can be made, one with low pH and one with a pH near neutrality so that the effect of low pH can be observed.

Pedagogical Issues

An investigation of microorganisms is developmentally appropriate for middle level students as well as high school students. Students at this level should know that microbes are everywhere—on dust particles and wood, on human skin, and in fish tank water. The experience of discovering just where microbes are found is important for more complex understandings about nutrition and energy flow later on (National Research Council 1996).

Students should also know that microbes are very small; too small to see with your eyes. To see them, biologists put them onto media and allow them to grow and form colonies. These colonies are composed of millions of individual organisms.

This simple but effective procedure for preparing microbiological media is a beginning point for each investigation. Once the students are familiar with the technique, they are able to expand their investigations into topics that are relevant and of interest to them. Such topics might include: the impact of environmental factors (e.g. temperature, pH, metals, radiation, etc.) on microbes; the effectiveness of various disinfectants, antiseptics, antibiotics, soaps and mouthwashes; enumerating the number of microbes in various sources (e.g. as in hamburger or on vegetables); and elucidating growth requirements for various microbes. Inquiry into student-generated questions promotes student curiosity and helps them develop the ability to ask scientific questions, to design and conduct investigations to answer their questions, and to use their observations to arrive at a reasonable explanation for the question posed. Students can identify items used in the home that help prevent the spread of bacterial infection (e.g. disposable cups, disposable tissues, disinfectants, antibacterial soaps) and equally important, discuss the *beneficial* contributions of bacteria.

This procedure provides an opportunity to collaborate with the consumer science (formerly known as home economics) teacher to teach kitchen cleanliness, effectiveness of hand and machine dishwashing techniques, effectiveness of hand washing (see Brown & Williams 1990 for procedure), and other health related issues, especially if one takes samples from the kitchen area. An interdisciplinary approach can be further expanded to include mathematical analysis of data and the exploration of plagues and their economic effects on society.

Conclusions

Students are intrigued and interested in learning more about microorganisms because they affect their daily lives. Even though studies show that science classes do not receive adequate support for supplies and equipment (Suter 1996), microbiology is accessible to classroom teachers. Low cost materials and adequate substitutes for expensive equipment can be used to successfully conduct inquiry-based investigations. Teachers can perform this procedure even if they lack expertise in microbiology.

It is important that students engage in scientific inquiry. The inclusion of laboratories and hands-on activities in science classrooms is a goal of many science teachers. This goal is supported by the National Science Education Standards' emphasis on inquiry-based teaching and learning. Indeed, the motivation for developing this procedure came from a middle level student who was investigating a possible science fair project involving microbiology. We hope this procedure will enable you to engage students in posing microbiological questions and pursuing the answers to those questions.

Acknowledgment

We would like to thank one of the anonymous reviewers for suggesting the inclusion of skim milk agar, V-8™ agar, and fruit and vegetable juice agars.

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Fermentation, Respiration & Enzyme Specificity

A Simple Device & Key Experiments with Yeast

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The study of fermentation and respiration by yeast is a powerful tool for presenting important aspects of metabolism to students. We will describe, in this article, a simple fermentation chamber and key experiments that give meaningful insights into metabolic processes.

A number of instruments have been used in student laboratories to quantify CO_2 production during microbial alcoholic fermentation. These devices include U-shaped fermentation tubes, manometers, nestled test tubes or vials (Heidemann 1985; Helms & Helms 1989), inverted pipettes (Yurkiewicz et al. 1989), and a water displacement apparatus (Tatina 1989). Although another fermentation device may seem to be redundant, the existing systems variously present problems with volume measurement, foaming, leaks, expense, long lag times and cumbersomeness. We will describe a simple, inexpensive and versatile fermentation tube that allows experiments to be conducted in as little time as 15 minutes.

Unfortunately, many fermentation experiments simply measure alcohol or CO_2 production without considering why this process takes place or its relation to other catabolic steps. We will present experiments that provide the background for productive discussions of the nature of catabolism, bioenergetics and metabolic controls. Investigations will also be described that take advantage of the recent availability of carbohydrate cleaving enzymes in pharmacies and grocery stores. The use of these enzymes can be coupled with fermentation studies to vividly demonstrate enzyme specificity and also serve as the basis for discussion of human digestive disorders.

Fermentation Tube & Basic Procedure

The fermentation tube is simply a plastic, 15-ml centrifuge tube (Corning #25319) that has volume gradations and a plug-type, screw-on cap (Figure 1). The top of the cap was perforated with three or four small holes (we used a 21-gauge hypodermic needle; a sewing needle or insect pin would work as well). Other brands of centrifuge tubes with plug-type caps should also work.

1. Prepare a 7% suspension of baker's yeast (one package Fleischmann's active dry yeast in 100-ml tap water) and appropriate sugar solutions (described later). Due to settling, the yeast suspension should be agitated immediately before use.
2. Fill a tube halfway (8 ml) with sugar solution and the remainder of the tube with 7% yeast suspension. When filling, extend the fluid meniscus above the lip of the tube.
3. Screw on the cap (a few drops will spurt from the holes) and confirm that any sizable air bubbles have been excluded.
4. Invert the tube and place in a large beaker filled with 40° C water. Place this beaker in a water bath to maintain a constant temperature. (The fermentation tube ejects yeast solution; the beaker prevents fouling of the water bath.)
5. At appropriate time intervals, mark the bottom of the CO_2 bubble (include any foam as part of the bubble) with a permanent, fine point marker. Periodically, the tube should be mixed by gentle inversion. An ample supply of fresh markers should be available.
6. At the end of the experiment, empty the tube and record the ml of CO_2 that correspond to the pen markings.

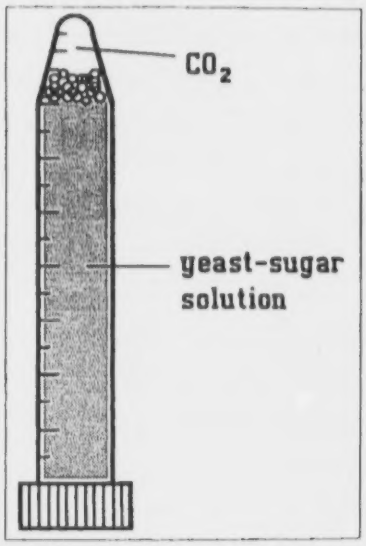

Figure 1. Fermentation tube made from a 15-ml, plastic centrifuge tube. Four perforations were made in the cap with a needle. After filling the tube with yeast and sugar solutions, the tube is capped, inverted and placed in a water bath. As CO_2 is formed, the yeast-sugar solution escapes through the perforated cap.

This fermentation tube and basic procedure have a number of advantages that include simplicity, low cost and flexibility. Measurements are easily made on multiple tubes at time intervals as little as one minute. The tapered tip of the tube allows for precise detection of small gas volumes, and fermentation is easily detectable within several minutes. These experiments will proceed even faster if the solutions are pre-warmed to 40° C. The relatively large volume of fluid also permits small samples of ferment to

be sampled for analysis. All of these factors allow a variety of experiments to be conducted in a limited time period.

Figure 2 depicts an experiment that used 0.5% glucose (w/v) as the initial sugar solution. At five-minute intervals the gas bubble was marked, the cap removed and glucose concentrations were determined using glucose test strips (Dextrostix®, Ames, CO.). In this study 0.5% glucose was used to match the upper sensitivity of the test strips. Lilly TES-TAPE®, relatively inexpensive and available at pharmacies, can also be used for this type of experiment; however, the initial concentration of glucose should be increased to 1%. Even this simple experiment provokes some interesting questions and suggests further experiments. What is the mechanism for continued CO_2 production after glucose depletion? Is the amount of CO_2 produced consistent with a fermentative process? (Ideally, each mmole of glucose should produce 2 mmoles CO_2 in an anaerobic environment.) How might temperature, pH or ions affect the rate of fermentation? Does ethyl alcohol inhibit fermentation? Which sugars can be used as substrates for fermentation?

One disadvantage of this fermentation tube occurs while attempting to read the indistinct volume markings on the Corning centrifuge tube. This problem can be easily remedied by providing students with a "reader card" that has volume markings. (A reader for Corning 15-ml tubes will be supplied by the authors on request.) This card can be held against the tube, and the marks made on the tube during the run can be easily converted to quantitative values.

Another potential problem is created by the ejection of the yeast and sugar solution through the holes at the bottom of the tube as CO_2 is produced. This problem results in underproduction of CO_2 at the end of the run. This can be corrected by dividing the amount of CO_2 produced in a time period by the remaining volume of yeast solution (i.e. ml $CO_2 \cdot min^{-1} \cdot ml^{-1}$ yeast solution). For most purposes, however, this correction is not necessary (compare Figures 3 and 4).

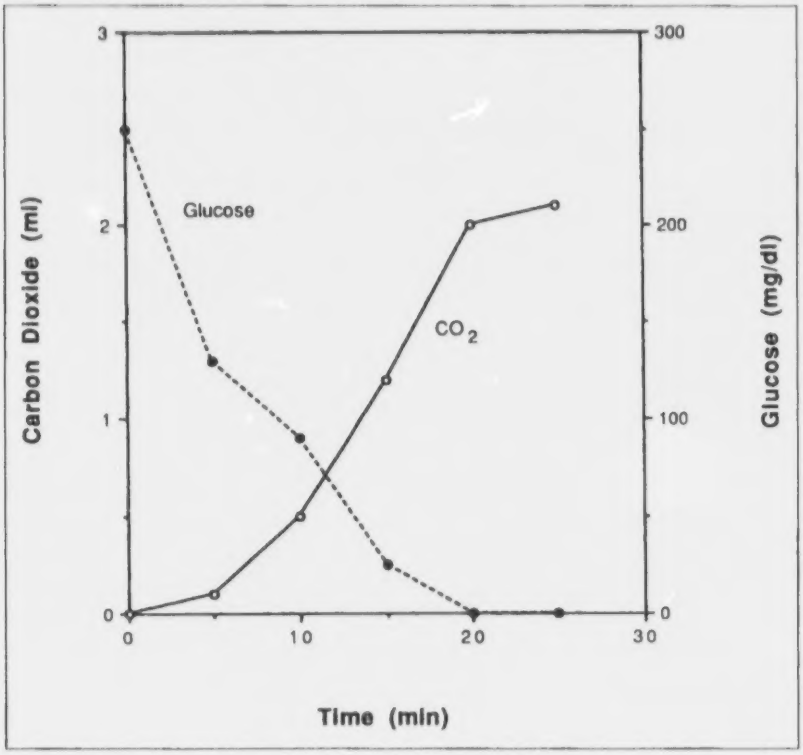

Figure 2. Carbon dioxide production and glucose utilization in a fermentation tube at 40° C. Equal volumes of 7% yeast and 0.5% glucose were added to to the tube. Glucose concentrations were determined by momentarily uncapping the tube and dipping a glucose test strip (Dextrostix®) into the fermenting solution.

Why Fermentation?

The fundamental function of fermentation is often overlooked in a basic biology course. During anaerobic conditions, the inhibition of mitochondrial adenosine triphosphate (ATP) synthesis results in high levels of NADH (and low levels of NAD^+) in the cytoplasm. ATP production must come from glycolysis but a shortage of cytoplasmic NAD^+ slows this pathway. Fermentation is a "side step" that regenerates cytoplasmic NAD^+ (Sols et al. 1971). Ethyl alcohol and CO_2 (or just lactic acid in animal cells) are byproducts.

Pasteur Effect

When asked about what would happen if the yeast and sugar solutions were bubbled with oxygen, most students (and many lab instructors) respond that CO_2 production will greatly increase. In reality, the opposite occurs.

Aerobic respiration of glucose can be summarized as follows:

$$C_6H_{12}O_6 + 6\ O_2 \Rightarrow 6\ H_2O + 6\ CO_2.$$

Optimally, most eucaryotic cells can produce about 36 ATP molecules during this process (Storey 1992). In other words, one ATP is associated with the release of 1/6 of a molecule of CO_2.

Baker's yeast, under anaerobic conditions, ferments glucose to ethyl alcohol and carbon dioxide:

$$C_6H_{12}O_6 \Rightarrow 2\ C_2H_5OH + 2\ CO_2.$$

Energetically, this is a far less efficient process; only two ATPs are produced per glucose. In terms of CO_2, one ATP is coupled to the production of one CO_2, six times the amount seen in respiration.

The critical point in considering the above is that catabolism, including glycolysis, is regulated, to a great extent, by the cell's available pool of ATP. Key metabolic enzymes, such as phosphofructokinase in glycolysis, are inhibited by high ATP levels and are activated by high levels of Associated Mitochondrial Products (AMP). AMPs such as citrate enhance ATP's inhibitory action (Stryer 1988). Thus, during fermentation, the metabolic "stops" have been pulled and glucose is rapidly (but inefficiently) consumed, releasing relatively large amounts of CO_2.

Pasteur first noted this inhibition of alcoholic fermentation by oxygen. In

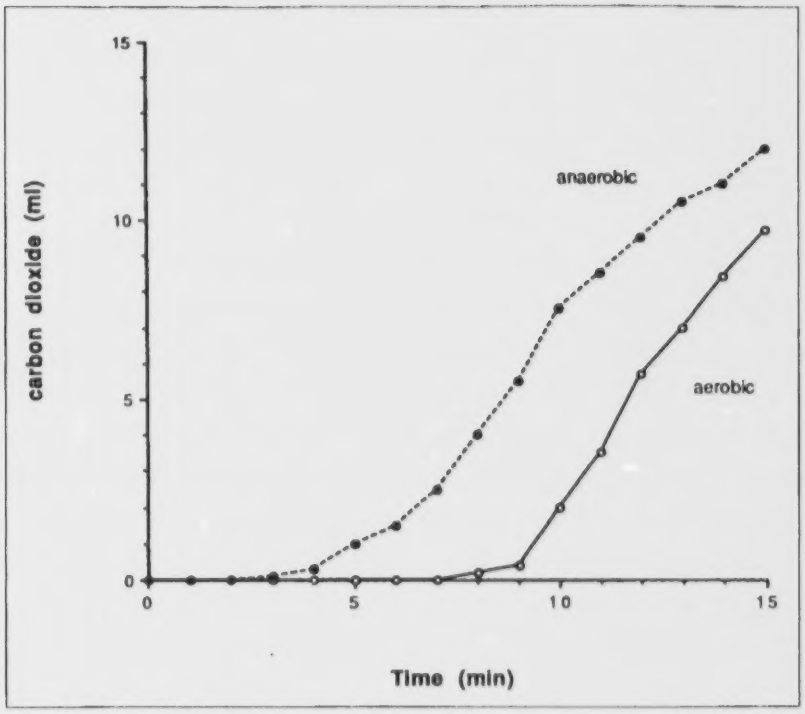

Figure 3. Carbon dioxide production under aerobic and anaerobic conditions at 40° C. Equal volumes of 7% yeast and 10% glucose were added to the fermentation tubes. The "aerobic" yeast and glucose solutions were bubbled with oxygen for several minutes before being added to the tube. The "anaerobic" yeast and glucose solutions received no special treatment.

fact, the "Pasteur effect" became a long-standing puzzle that eventually led to our current knowledge of glycolysis and the nature of metabolic regulation (Sols et al. 1971). In our opinion, an experiment dealing with the Pasteur effect ranks near the top of the "must do list" for biology students. Such experiments are simple, inexpensive and can lead to very productive discussions concerning bioenergetics and the nature of metabolic regulation.

The following simple experiment demonstrates the Pasteur effect:

1. Separately, bubble a 10% glucose solution and a 7% yeast suspension with oxygen for several minutes. Designate these as the "aerobic" mixtures.
2. Prepare a second set of 10% glucose and 7% yeast mixtures that have not been bubbled and designate them as the "anaerobic" mixtures.
3. Place 8 ml of "aerobic" glucose and 8 ml of "aerobic" yeast mixtures in a fermentation tube. Prepare a second tube containing the "anaerobic" mixtures.
4. Cap both tubes, invert and place in a 40° C water bath as described in the previous experiments.
5. At one-minute intervals, mark the bottom of the gas bubble; allow the experiment to run for 15 minutes.

Figure 3 depicts the results of one such experiment; Figure 4 is a volume-corrected version. Similar results, but to a slightly lesser degree, are obtained if the "aerobic" yeast and sugar mixtures are aerated with an aquarium pump rather than bubbled with oxygen. Bubbling with nitrogen enhances the anaerobic response but this effect is diminished if excess stirring of the yeast occurs.

During analysis of these experiments the rates of CO_2 should be determined: Do the anaerobic yeast, at any point, have a six-fold production in CO_2 as compared to the aerobic yeast? Students might also measure the rates of glucose consumption in these experiments and compare their findings to theoretical expectations. Additional twists to the above experiment in advanced courses might include the addition of ATP, ADP or AMP salts, citrate (an important metabolic feedback signal) or dinitrophenol (which diminishes the aerobic ATP pool by uncoupling oxidative phosphorylation).

Fermentation, Enzyme Specificity & Digestive Disorders

Two enzymes that are relatively new, "over-the-counter" pharmaceuticals can be combined with fermentation studies to illustrate enzyme specificity and are an interesting way to introduce the topic of human digestive disorders.

Lactose intolerance, the inability to digest milk sugar, is a prevalent clinical disorder. In certain ethnic groups, such as Asians in the U.S., all adults have some degree of lactose malabsorption (Büller & Grand 1990). Individuals with this condition have stopped producing the enzyme lactase (a beta-galactosidase) which is needed to hydrolyze lactose, a disaccharide comprised of "beta-linked" galactose and glucose (Figure 5). Fortunately, these people can add commercially prepared lactase (e.g. Lactaid® or Dairy Ease®) to dairy foods to reduce their digestive misery.

Foods such as beans and cabbage that contain the oligosaccharides raffinose and stachyose are another source of digestive difficulty for most people (Wardlaw & Insel 1990). These sugars are incompletely digested by humans but are avidly consumed by gas-producing bacteria in the large intestine. A new consumer product, Beano® (AkPharma, Inc.) promises to deflate this age-old problem. Beano® is an alpha-galactosidase that cracks the troublesome portion of these sugars (alpha-linked galactose-glucose; the disaccharide with this configuration that reaches the large intestine is melibiose). Several drops of Beano® on a serving of offending food greatly diminishes gas production.

Baker's yeast is unable to metabolize lactose or melibiose (Suomalainen & Oura 1971), which creates an ideal experimental opportunity. The following experiment demonstrates the enzymatic modification of "non-digestible" disaccharides and enzyme specificity:

1. Obtain Lactaid® drops (Lactaid, Co.) and Beano® drops (AkPharma, Inc.) at a pharmacy. Although not tested, Dairy Ease® drops (Winthrop Consumer Products) seem to be equivalent to Lactaid®.

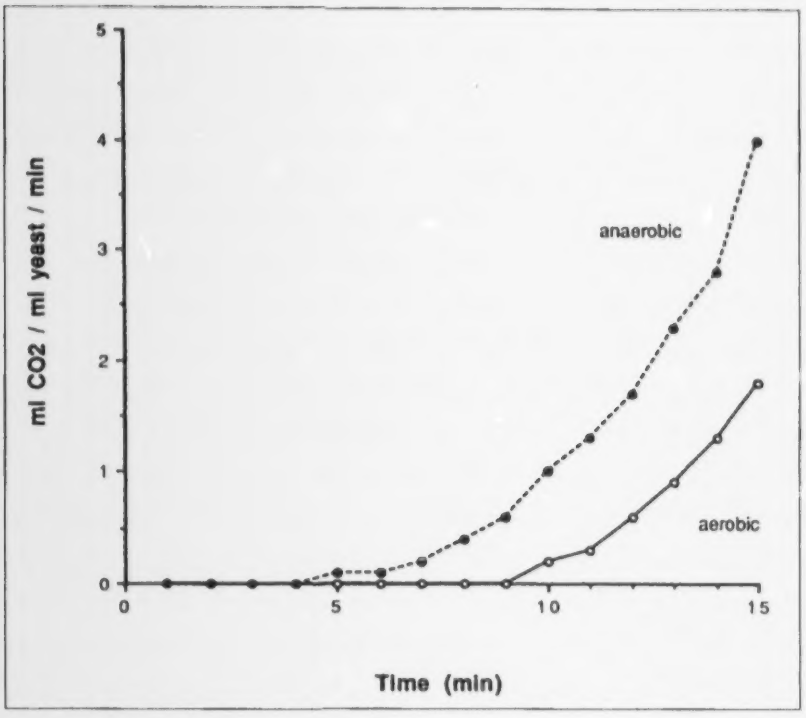

Figure 4. Data from Figure 3 corrected for loss of yeast-sugar solution as a result of CO_2 production.

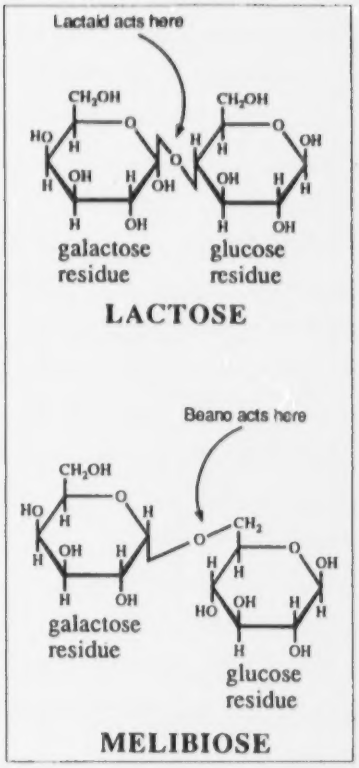

Figure 5. Actions of galactosidases. Lactose is specifically hydrolyzed by lactate (Lactaid®), a β-galactosidase. Melibiose contains the same monosaccharides as lactose but has an α-galactosidic bond. Beano® contains an α-galactosidase that specifically attacks this second type of bond.

2. Prepare solutions of 5% lactose (β-D-Lactose, Eastman Kodak Co. #114 7040) and 5% melibiose (Sigma Chemical Co. #M-5500).
3. Label six fermentation tubes 1 through 6.
4. Transfer 8 ml of 5% lactose to Tubes 1, 2 and 3 and then add the following:
   Tube #1—no further additions
   Tube #2—2 drops Lactaid®
   Tube #3—2 drops Beano®
5. Transfer 8 ml of 5% melibiose to Tubes 3, 4 and 5 and then add the following:
   Tube #4—no further additions
   Tube #5—2 drops Lactaid®
   Tube #6—2 drops Beano®
6. Mix the tubes by swirling and allow them to set for 10 minutes.
7. After this 10-minute period, fill the remainder of the tubes with 7% yeast, cap, invert and place in 40° C water bath.
8. After 20 minutes in the water bath, mark the bottom of the CO_2 bubble and record the results.

The results of such an experiment appear in Table 1. As can be seen, these nonmetabolizable sugars are transformed by the appropriate enzymatic treatment. Furthermore, a high degree of enzyme specificity is demonstrated; virtually no cross reactivity is seen between these alpha and beta galactosidases.

Melibiose is a somewhat expensive sugar; the concentration can be reduced to 1% and an effect will still be seen, although less pronounced. Alternatively, raffinose (galactose-glucose-fructose) can be used. Raffinose is partially metabolized by yeast; the rate of fermentation increases with the addition of Beano®.

The apparatus described in this paper is well-suited for introductory laboratories, especially when simplicity and time are important considerations. The versatility of this device and the associated procedures also lend themselves well to projects and advanced courses. For example, a series of experiments could be designed to "dissect" lactose metabolism: Will disrupted yeast cells ferment lactose (i.e. is there a permeability problem)? Is lactose metabolism inducible in baker's yeast? Does digested lactose (i.e. Lactaid®-treated) produce enough CO_2 to account for both sugar units? Are both glucose and galactose metabolized? Such studies are fertile grounds for the formulation and testing of hypotheses.

Table 1. Fermentation of 5% lactose and 5% melibiose by baker's yeast and the influence of Lactaid® and Beano®. CO_2 volumes were measured after 20 minutes of incubation at 40° C.

Tube No.	*Contents*	*CO_2 produced (ml)*
1	lactose	0
2	lactose + Lactaid	11.0
3	lactose + Beano	0
4	melibiose	0
5	melibiose + Lactaid	0
6	melibiose + Beano	2.0

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Fun Microbiology

Making Root Beer & Other Naturally Carbonated Beverages

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Everyone likes an ice-cold soft drink or soda pop, especially on a hot summer day. Almost every gas station, convenience and/or grocery store will carry some commercial brand of soft drink on its shelves. These beverages are prepared by adding a flavor concentrate (syrup) and sugar to water, which is then mixed and carbonated under pressure with CO_2 gas. Soft drinks have been made by this same general procedure for the past 100 years (Dietz 1973; Riley 1972; Simmons 1983).

Only a limited number of flavors (brands) are commercially available, based primarily on production costs and consumer demand. Someone interested in preparing a soft drink to suit one's own taste might try combining a flavor concentrate (commercial or homemade) and sugar with soda water (club soda) for the carbonation (Johnson 1992). A more interesting (and original) method would be to allow yeast to carbonate a soft drink through the natural fermentation process.

Many people (including myself) prefer the "richer" flavor associated with the naturally carbonated homemade soft drink over the commercial store brands. An unlimited number of flavors are possible by mixing varied combinations/types of syrups, herbs and sugar sources. Prior to large-scale commercial production, people would collect locally available ingredients to prepare naturally carbonated soft drinks including spruce, birch, nettle, sassafras (sarsaparilla), honey, and molasses plus ginger (switchel) to name a few (Riley 1972).

The yeast *Saccharomyces cerevisiae* (baker's yeast) was the most commonly used microbe and is still used today to produce many naturally fermented products including bread, certain beers and wines, and single-cell protein (Jay 1992; Prescott, Harley & Klein 1993; Prescott & Dunn 1983). In the absence of oxygen (anaerobic), this yeast ferments simple sugars to carbon dioxide (CO_2) and ethanol. The CO_2 is produced as small gas bubbles which "carbonate" liquids within sealed containers, or lighten (leaven) the texture of dough causing it to rise. The ethanol produced remains in liquids (unless distilled-off), but evaporates during the baking process in breads. This yeast also imparts a characteristic flavor to the fermented product with a taste we also enjoy.

In this experiment, the yeast is provided with the disaccharide sucrose (table sugar) as a source of energy. This sugar is first broken down into a molecule each of glucose and fructose by the yeast enzyme invertase. These simple sugars then enter into the central metabolic pathway glycolysis, which is one of several pathways known generically as anaerobic fermentations, by which many organisms extract chemical energy from various organic fuels in the absence of oxygen (Lehninger 1975).

In animals, glycolysis serves as an important emergency mechanism capable of yielding energy for short periods when oxygen is not available (e.g. strenuous exercise). In this situation, glycolysis' end-product pyruvic acid is converted to lactic acid which is the molecule that causes the aching of muscles. Under normal living conditions (aerobic), animals convert the pyruvic acid from glycolysis to acetyl-CoA which then enters the Krebs or tricarboxylic acid cycle to eventually produce energy in the oxidative phosphorylation process. Yeast fermentation is limited to the same enzymatic pathway as glycolysis by forming pyruvic acid, but requires two different enzymatic steps at the end. A summary of the yeast fermentation pathway is shown in Figure 1. For each mole of sucrose metabolized by the yeast, potentially four moles each of CO_2 and ethanol are formed as products.

Within the time frame allowed for in the following experiments (8–48 hours) and the cocktail mixtures tested, very little ethanol is produced in homemade fermentations. Final alcohol (ethanol) content will range from trace amounts to 0.5% (v/v), depending upon how nutritious the cocktail mixture may be (e.g. root beer concentrate plus sugar is less nutritious than honey). Commercial American beer (e.g. pilsner) has an ethanol content of 5.1% (v/v).

Objectives of this study were to:

1. Determine which commercial syrups produce the best-flavored naturally fermented soft drink(s).
2. Test varying amounts of selected commercial syrups to optimize flavor.
3. Produce a low-calorie, naturally fermented soft drink.
4. Attempt to develop a new flavored soft drink.

Root Beer & Other Flavor Syrups

Commercial flavor concentrates of McCormick® (root beer) and Homebrew® (root beer, birch root beer, sarsaparilla, cola, strawberry and ginger ale) brands were diluted according to label directions in 1 gallon of water. Table sugar (2.5 cups) and dried Fleischmann's® yeast (1 teaspoon) were added and the different cocktails thoroughly mixed and poured into clean 20-ounce plastic screw cap bottles (four bottles per cocktail mixture). Lids were tightly sealed and bottles were incubated in an upright position at room temperature (23-27° C) for 48 hours for sufficient carbonation to occur. When using plastic bottles, one may easily check for carbonation (without having to open the bottle) by squeezing. If the sides of the bottle do not give way, sufficient carbonation has occurred. Following carbonation, bottles were placed into a refrigerator for one week after which the different soft drinks were compared for pH, culture purity, and flavor qualities.

All soft drink cocktails where fermentation (carbonation) occurred exhibited a pH of 5.0 or less and contained only yeast cells, as determined by pH paper and microscopic examination, respectively. Homebrew "sarsaparilla" had no carbonation (even after allowing bottles to ferment an additional 24 hours) which suggests it contained ingredients that inhibited

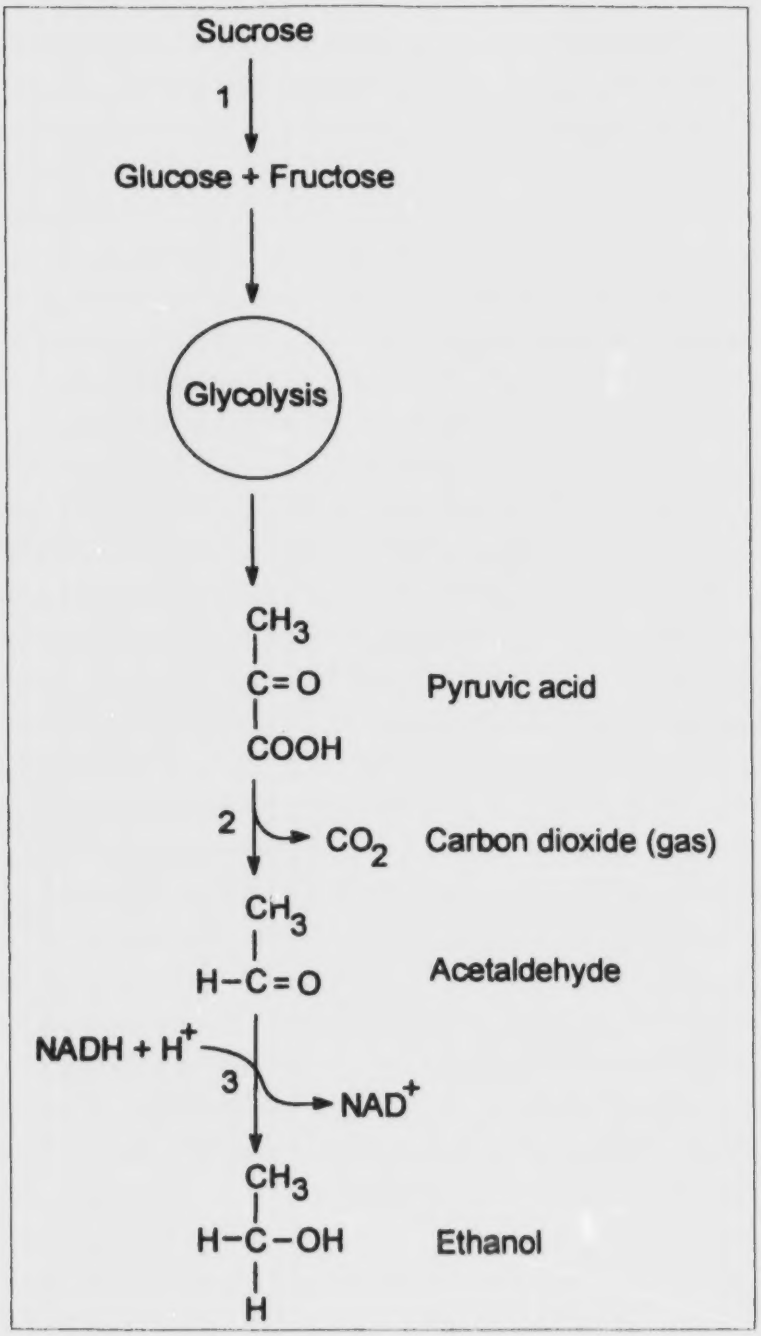

Figure 1. Yeast fermentation pathway. Unique enzymes labeled include: (1) invertase, (2) pyruvate decarboxylase, and (3) alcohol dehydrogenase.

natural yeast carbonation. All other Homebrew syrups tested produced soft drinks that exhibited an unpleasant aftertaste which students disliked. McCormick "root beer" produced a smooth, good-flavored soft drink with no lingering aftertaste.

The McCormick "root beer" soft drink produced by following label directions (2 oz concentrate/5 gal water = 0.87 Tbl/gal) resulted in a weaker-than-desired root beer flavor. Different amounts of syrup concentrate (1–4 Tbl/gal) were diluted and carbonated as described above to determine which concentration(s) would produce the best-flavored soft drink. Results of student taste tests were compiled and the best root beer recipe is presented in Table 1.

To optimize conditions for successful carbonation, one should adhere to the following precautions:

1. Use dried yeast prior to expiration date.
2. Mix cocktail containing yeast well before pouring into bottles.
3. All containers and utensils should be clean and composed of nonmetal material.
4. Fill bottles to within 2 inches below the rim to exclude oxygen which will lessen or inhibit fermentation and possibly encourage growth of spoilage microbes.
5. Tightly seal bottles.
6. Use nonchlorinated water.
7. Incubate bottles at 23–27° C (74–80° F) for 41–48 hours.
8. Drink immediately after chilling or store no longer than 7–8 days in a refrigerator.

It is important to remember that the yeast are still alive and therefore continue metabolizing the sugar (even within the refrigerator). Storage longer than one week may result in root beer with a "dry" or "unsweetened" flavor. One may also add lemon juice when preparing the cocktail mixture to lower

Table 1. Root beer recipe (makes almost 1 gallon).

14 cups (3316 ml) nonchlorinated water[a]
2–3 cups table sugar (sucrose)[b]
2–3 Tbl (27–41 ml) root beer concentrate[c]
1 tsp dried yeast[d]

[a] May use distilled water or tap water that has been allowed to stand 2–3 days.
[b] May substitute equal amount of honey for different flavor! For low-calorie root beer, use 0.5 cups sugar + 4.8 Tbl of Equal® sweetener.
[c] McCormick & Co., Consumer Affairs Department, 211 Schilling Circle, Hunt Valley, MD 21031.
[d] Add 1 tsp dried yeast (Fleischmann's®) to 40 ml nonchlorinated water and allow to stand 5–10 mintues prior to adding to above mixture. Incubate in sealed bottles 41–48 hours at room temperature (23–27° C).

Table 2. Combinations of table sugar (sucrose) and Equal® (NutraSweet®) added to 14 cups water.

Treatment	Table Sugar (cups)	Equal[a] Tbl (g)	# Calories per 20 oz.
1	0	6.0 (60.0)	0
2	0.25	5.4 (54.2)	44
3	0.50	4.8 (48.4)	88
4	1.00	3.6 (35.9)	176
5	1.50	7.25 tsp (24.2)	264
6	2.00	3.50 tsp (11.7)	352
7	2.50	0	440

[a] Tbl = tablespoon, tsp = teaspoon, g = gram, oz = fluid ounces

the initial pH to 5.0 or below. This additional precaution will inhibit growth of potential spoilage microbes (e.g. bacteria) if present, but still allow the yeast to carbonate.

The root beer recipe listed in Table 1 is just one variation that students developed in class. In your class, you may want to start with this recipe and try substituting table sugar with other types of sugars such as fructose or honey. My personal favorite is to sweeten the root beer cocktail with honey and to fortify flavor even more with vanilla extract. Feel free to experiment with your own sugar and flavor syrup measurements; you may come up with a better-flavored soft drink!

Low-Calorie Root Beer

Several combinations of table sugar amended with the sugar substitute Equal® (NutraSweet®) were prepared so that the total sugar content (sweetness) of 2.5 cups/14 cups water resulted (Table 2). A sweetness equivalent chart is presented in Table 3. This experiment was conducted to determine the minimum amount of metabolizable sugar (e.g. sucrose) required by yeast for sufficient carbonation of a low-calorie (diet) soft drink. Equal® sweetener was chosen because:

1. Its flavor is superior to other sugar substitutes.
2. It is commonly used in commercial diet soft drinks.
3. Yeast do not sufficiently metabolize (use or break down) this amino acid derivative.
4. No heating is required for natural yeast carbonation, a process which would destroy NutraSweet® and result in a nondesirable, unsweetened flavored soft drink. As the reader may know, NutraSweet™, or aspartame, is a sweet-tasting amino acid derivative used as a "non-calorie" sweetener because humans cannot metabolize this compound.

Sugar solutions (treatments) from Table 2 were each amended with 2.5 tablespoon McCormick "root beer" concentrate and 1 teaspoon dried yeast (Fleischmann's), bottled and tested as described previously. Carbonation occurred after 48 hours only within cocktail mixtures containing table sugar (0.25–2.5 cups). The least amount of table sugar (amended with Equal®) which resulted in a sufficiently carbonated soft drink of good flavor was 0.5 cups (88 cal/20-oz bottle). This is one-fifth of the recommended sugar rate of 2.5 cups (440 cal/20-oz bottle), and indeed, a low-calorie soft drink.

Table 3. Equivalent sweetness chart.

For this much sugar	Use this much Equal[a]
2 tsp	0.25 tsp[b]
1 Tbl	0.50 tsp
0.25 cup	1.75 tsp
0.33 cup	2.50 tsp
0.50 cup	3.50 tsp
1.0 cup = 232 g = 870 cal	7.25 tsp = 24.3 g
1 lb (~2.33 cups)	5 Tbl + 2 tsp = 2 oz = 56.7 g = 1 pouch

[a] Information taken from label on Equal® package
[b] Tbl = tablespoon, tsp = teaspoon, g = gram, cal = calories, oz = ounces (mass)

Sweet-Tart Soft Drink

Students were also challenged to develop a new soft drink flavor from easily accessible ingredients. Creative endeavors focused on various combinations of honey and citrus fruits and resulted in a product that exhibited a sweet-tart flavor (Table 4). Using lemons and oranges not only added the citrus tart flavor, but also adjusted (lowered) the pH of the cocktail to below 4.0, a value known to inhibit bacterial spoilage while allowing yeast fermentation. Honey is more nutritious to yeast than many commercial flavor concentrates (most of which include poorly nutritious artificial ingredients). Therefore, sufficient carbonation occurs within 18–24 hours incubation at room temperature with the sweet-tart recipe listed in Table 4. Care should be taken to not allow longer fermentation, or bottles might explode or foam excessively when opened.

Several recipes for producing naturally carbonated soft drinks have been presented. Ingredients for preparing these are inexpensive and may easily be found in a local grocery store. To optimize a successful soft drink carbonation by natural yeast fermentation in your class (using commercial or homemade ingredients), two factors are of utmost importance:

1. Always use sanitary conditions when following the above procedures.
2. When producing your own cocktail recipe, conduct a trial test to determine the proper incubation period for carbonation.

Nutritionally rich fruit juices (e.g. grapes, apples, peaches, etc.) will carbonate sooner than a cocktail composed of a commercial flavor syrup. For example, yeast will carbonate apple juice (containing no chemical

Table 4. Sweet-Tart soft drink recipe.

1 gallon (3.8 L) nonchlorinated water[a]
4 oranges (juiced)
4 lemons (juiced)
3.8 lbs honey[b]
1 tsp dried yeast[c]

[a] May use distilled water or tap water allowed to stand 2–3 days; L = liters.
[b] May use fresh hive honey or commercial bottled honey.
[c] Add 1 tsp dried yeast to 40 ml nonchlorinated water and allow to stand 5–10 minutes prior to adding to above mixture. Incubate in sealed bottles 18–24 hours at room temperature (23–27° C).

preservatives) within 6–12 hours when following the above procedures. The root beer recipe described in this paper will require at least 41–48 hours incubation for sufficient carbonation. Prior to drinking, it would also be advisable to ascertain that the product has a pH of 5.0 or less and only yeast cells are observed under the microscope.

This paper also summarizes results of the first laboratory exercise I conducted in my applied microbiology class. A major portion of the lab section challenges students to both design and conduct experiments to solve specific problems associated with microbiological production. In this exercise, students are asked to produce a naturally carbonated soft drink of comparable flavor/quality to a commercial brand. I use this exercise to introduce students to both the cooperative learning process and the use of the scientific method of question solving. With root beer as a model system, students rapidly learn the practical importance of replication, data collection and proper control treatments when conducting experiments. As with many scientific endeavors, more questions than answers are developed. Upon completing the root beer recipe, questions invariably arise as to whether a diet soft drink can be produced by natural fermentation. Free-thinking and creativity are taking hold, and the class has interesting discussions on microbe metabolism and how to approach a low-calorie soft drink. By this time, students are more comfortable in experimental design and by the next lab period want to try to develop their own soft drink flavor!

This exercise is not just limited to the field of biology. Other science content areas, including chemistry and physics, play an important role in successful root beer fermentation. A potential chemistry project might involve the student trying to demonstrate how many moles of ethanol and CO_2 are produced when the yeast is presented with either a monosaccharide (e.g. glucose) or a disaccharide (e.g. sucrose) as an energy source. Fermentation flavor qualities based upon the type of sugar or flavor syrup used might also be investigated by individuals interested in food science. A physics project might investigate the effect of CO_2 concentration (pressure) on growth rate of yeast in the root beer cocktail. Dependent upon incubation temperature and ingredients of the cocktail mixture, yeast growth is inhibited at high CO_2 concentrations. Sugar fermentations are carried out by yeast cells at CO_2 pressures ranging from 0.48–14 atmospheres (ATM) or 7.0–205.8 pounds per square inch (psi) (Prescott & Dunn 1983). Whichever field of science you investigate with your class, the root beer experiment is a fun, problem-solving and knowledge-gathering experiment with an underlying applied aspect. Good luck with your own fermentation project!

Acknowledgment

Gratitude is extended to Lesslie Coffin for typing this manuscript.

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Fun Microbiology

How to Measure Growth of a Fungus

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Microbiology is the study of microorganisms, a large and diverse group of organisms that exist as single cells or cell clusters, which are individually capable of carrying out all life processes of growth and reproduction. The extreme importance of microbiology to the world cannot be overemphasized. Many students have the impression that microbes (e.g. bacteria, fungi and protozoa) only cause disease and they fear studying them (Williams & Gillen 1991; Gillen & Williams 1993). However, society greatly benefits from microorganisms in many ways. They are necessary for the production of bread, cheese, yogurt, beer, wine, antibiotics, vitamins, vaccines, enzymes, food supplements, and many other important products. The rapidly expanding field of biotechnology rests upon a microbiological foundation (Prescott et al. 1993). Furthermore, microbes play an essential role in defining the ecosystem by acting as a primary source of nutrients at the base of all ecological chains, as well as by playing an important role in the biogeochemical cycling of carbon, oxygen, nitrogen, phosphorus and sulfur.

Almost every investigation involving microbes will require a method of monitoring their growth. Several articles have been published on how to measure the growth of bacteria (Ken-Youn & Torres 1993; Paulton 1991), protozoa (Jones 1980), and algae (Lynch & Fenwick 1974; Martinez & Eugenie 1991; Steedman 1980) in a teaching situation. Little information is found in the literature describing a method of monitoring the growth of a fungus in a classroom situation. Since the importance of microbes to our everyday lives is already recognized, we as educators should present students with a variety of different types of microbes which we benefit from, including the fungi.

The purpose of this experiment is to demonstrate a simple method of measuring fungus growth. Specifically, it is to monitor the effect of temperature on the growth of *Trichoderma viride*. This fungus was chosen as a model system because it:

1. Is a nonpathogenic fungus widespread in soil throughout the world
2. Exists as a saprophyte (lives upon nonliving organic material)
3. Is currently under investigation for potential use as a biological control agent against plant pathogens (Cook & Baker 1983)
4. Has a very rapid growth rate at conducive temperatures.

Materials & Methods

Trichoderma viride inoculum can be produced in two 15 × 100 mm plastic Petri dishes (plates) containing potato-dextrose agar (PDA). Plates should be incubated at room temperature (23–27° C) until the perimeter of the fungus colony almost reaches the outer margin of the dishes. This fungus grows radially on the aerobic surface of a solid medium (e.g. PDA) producing a white filamentous (cottony) mycelium when young, which later turns to a dark green color as the vegetative stage matures to porduce spores or conidia. An alcohol (95% ethanol) sterilized 6-mm brass cork-borer can be used to aseptically cut circular disks (plugs) of the fungus inoculum from the white perimeter portion of the colony. This region of the colony represents the youngest growth of a similar age and can be used as a standardized inoculum for experimental plates. A plastic drinking straw, or thin-walled hollow glass tubing, previously dipped in alcohol can also be used to cut the disks. An alcohol-sterilized cork-borer can be passed through a flame to remove the alcohol prior to cutting the plugs. A flame-sterilized dissecting probe can then be used to aseptically pick up and transfer one disk containing the fungus to the center of a fresh (experimental) 15 × 100 mm PDA plate. Care should be taken to prevent any further damage to the fungus (upper surface) portion of the plug. The disk should be inverted so that the fungus comes into direct contact with the fresh medium. Experimental plates inoculated in this manner can be incubated at 6, 12, 22, 27, 30, 34 and 37° C. Four plates at each temperature are sufficient. The surface diameter of the fungus colony grown in each inoculated dish can be measured periodically until the colony reaches the outer margin of the plate. Time (in hours) and average colony diameter (in mm) can then be plotted for each temperature. Respective growth rates (slope = mm/hr) can be calculated from the equation for a straight line ($y = mx + b$) for each temperature tested.

Results

Measurements of *T. viride* growth at different temperatures can be recorded at 17, 26, 42.3, 50.3, 67.7 (12–37° C), and 211.3 (6° C) hours after inoculation. When recording growth, subtract the diameter of the inoculum plug (e.g. 6 mm) from each measurement. For example, if the average colony diameter after 17 hours at 22° C from 4 plates is 13 mm, the actual growth is 7 mm (13 mm − 6 mm).

Time (x) and average colony diameter (y) values can be analyzed by linear regression. The best-fit line for each temperature, including standard deviations (error bars) for each set of data, is presented in Figure 1. Growth of this fungus (and most other filamentous fungi) follows a linear function or model on a solid medium such as PDA. The growth rate (mm/hr) of *T. viride* is represented by the slope of the line obtained for each temperature (Figure 1).

Validation of this model is presented in Table 1. The correlation coefficient (r) values were very high for all temperatures tested where growth occurred. A perfect correlation between two

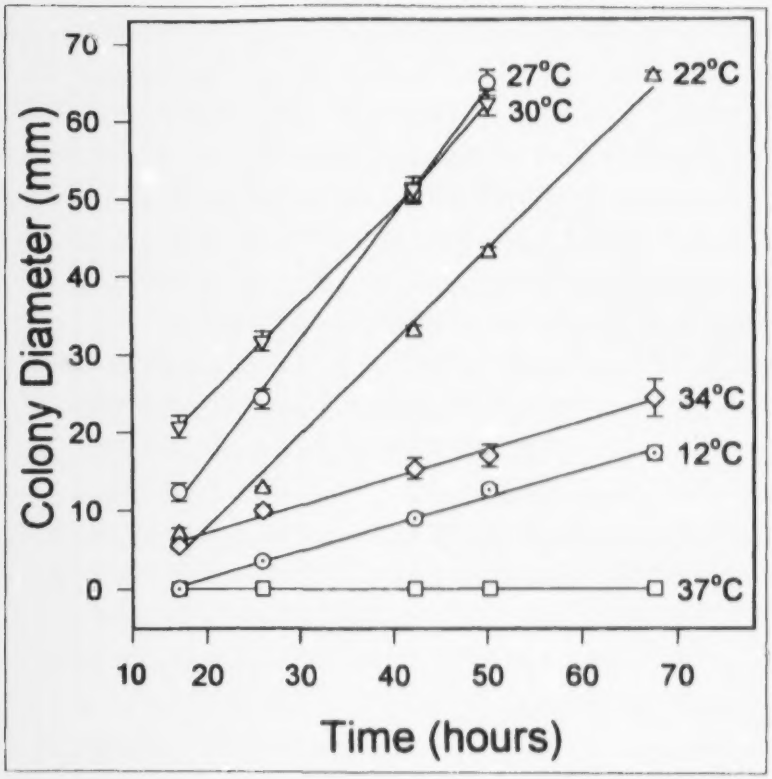

Figure 1. Growth of *Trichoderma viride* at selected temperatures on potato-dextrose agar medium. The slope of each line represents the growth rate at each temperature.

Table 1. Linear regression analysis of the effect of temperature on growth of *Trichoderma viride* on PDA.

Temperature (°C)	*Y-intercept*[a] *(mm)*	*Slope (mm/hr)*	*r-value*
6	1×10^{-11}	0.020	0.99
12	−5.540	0.344	0.99
22	−15.70	1.180	0.99
27	−15.60	1.580	0.99
30	−0.410	1.236	0.99
34	−0.028	0.356	0.97
37	no growth		

[a] Equation for a line: y = mx + b; where b = Y-intercept and m = slope.

variables (e.g. time and colony diameter) is an r-value equal to 1.0 (−1.0 is a perfect negative correlation). In other words, by knowing the value of one variable, the value of the other variable can be predicted with 100% accuracy. The further the r-value is from 1.0, the less reliable any predictions will be. Temperature vs. growth rate values obtained from Table 1 are plotted in Figure 2. This provides an easier method of observing the optimal temperature for growth of this fungus. Optimal growth of *T. viride* on PDA occurs at 27° C. This produces a graph slightly skewed to the left, which is a characteristic response for the effect of temperature on growth and development of a fungus (Mitchell 1984).

Discussion

This experiment is currently conducted by students in the general microbiology class offered at Ball State University. The variable of temperature was chosen because most classrooms or laboratories have access to several different temperature environments (e.g. refrigerator, ambient and incubator). A culture of *T. viride* and prepared plates of potato-dextrose medium are available through major biological supply companies. *Gliocladium virens* (= *T. virens*) and most *Penicillium* species are other rapidly growing nonpathogenic fungi that could be substituted for *T. viride*. The growth rates of each of these fungi might be compared. If there is access to only one temperature in your classroom, the growth of *T. viride* on different agar media (e.g. yeast malt, nutrient, Sabouraud-dextrose, potato-dextrose, oat flake, etc.) could be investigated. More options are possible if an autoclave or pressure cooker is available for preparing customized media. In this latter situation, media to investigate the effects of pH, salts, and/or chemical preservatives on the growth of a fungus could be prepared. Whichever of the above parameters you choose to investigate in your class, utilizing *T. viride* should assure experiment completion within 2–3 days.

Monitoring growth of a filamentous fungus on a solid or agar-amended medium such as PDA has many advantages. Growth measurements are very easily taken by students (only a ruler is needed) and can be completed in a minimal amount of time. By using a standardized inoculum (plugs) there is little variation between replicates (plates) as evidenced by the very small standard deviation values or error bars shown in Figure 1. Petri dishes are not very large. A sufficient number can be provided for each student to record measurements, and they do not take up a lot of space. Growth of a filamentous fungus on a solid medium is linear. This type of mathematical function or relationship is easy to use and teach. Linear regression analysis (Table 1) of data collected in this experiment produces very high r-values, suggesting that very accurate data readings can be collected. This provides additional support for the dependability of this experiment to provide successful and useful data for classroom demonstrations. It should be noted that several of the best-fit lines from Figure 1 do not intersect at 0.00. The Y-intercept values are not equal to 0.00 (Table 1) especially at the more conducive growth temperatures. This is most likely due to damage of fungus mycelium when cutting the inoculum plugs. The initial growth lag-time is more evident at temperatures conducive to the outgrowth of this fungus. What is most important is that once growth begins, it is linear with time as Figure 1 demonstrates.

Another important advantage of using this experimental design for demonstrating growth of a fungus is that it allows students the opportunity to collect data and present their results in a scientifically precise way. The experiment is designed well, and sufficient data can be collected to answer the hypothesis: "What is the optimal temperature for growth of this fungus?" Plotting the results of temperature vs. growth (as shown in Figure 1) is a good 'preliminary' tool for looking at how well data can fit a mathematical model (e.g. linear function).

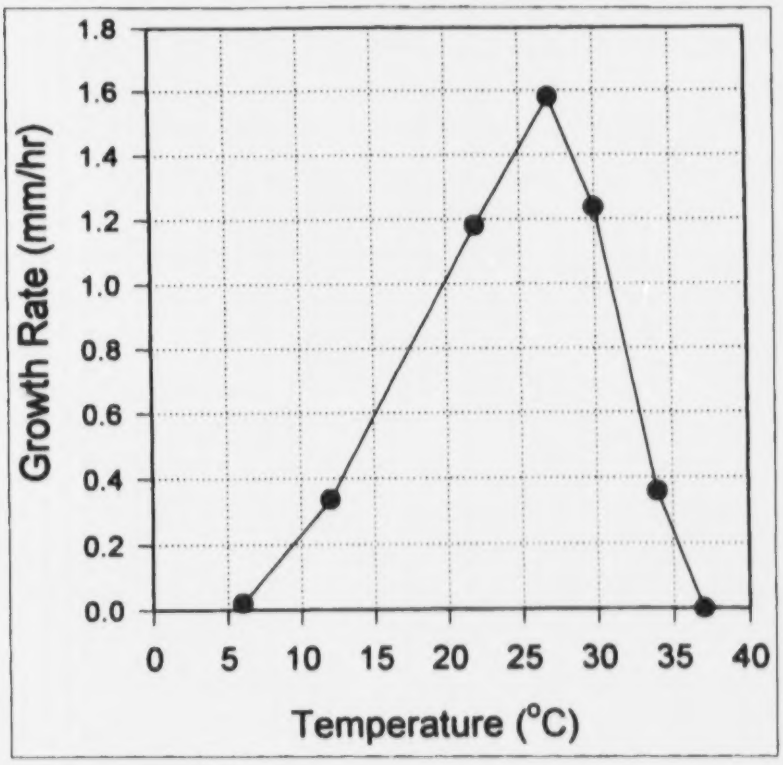

Figure 2. Effect of temperature on growth rate (mm/hr) of *Trichoderma viride*.

However, students must provide an alternative method of presenting their results so that the optimal temperature for growth of this fungus is more evident. This experiment teaches students to utilize primary information and to transform it into a more descriptive unit of measurement: growth rate (= mm/hr); which is then presented in the manner shown in Figure 2.

This is also a good experimental model to introduce students to the importance of using the computer as a scientific tool both for analyzing and presenting data. If a computer is available, students can learn to use graphics software, basic statistical analysis, and the importance of replication of treatments. Hopefully your students will have as much fun as ours do in monitoring growth of a filamentous fungus!

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Acetobacter xylinum
An Inquiry into Cellulose Biosynthesis

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Cellulose is the most abundant natural polymer on Earth. It is the major constituent of higher plants, and cellulose fibers have important industrial applications including the clothing that we wear and even the paper upon which this article is written. Cellulose forms the cell wall of eukaryotic plants, algae and fungi, and is synthesized by a variety of bacteria, including *Acetobacter xylinum*. *A. xylinum*, a Gram-negative bacterium found in the soil, can frequently be isolated from decaying fruit such as apples that have fallen to the ground. In addition to serving as a model organism for the study of cellulose synthesis, *A. xylinum* can serve as an interesting and instructive organism in the classroom to help students understand the process of science. The major focus of this article will be upon how students can experiment with *A. xylinum* using an inquiry approach to their learning.

Acetobacter xylinum is an aerobic soil bacterium in the family of bacteria that ferments carbohydrates to vinegar (*A. aceti*). *A. xylinum* is an unusual member of this family because it synthesizes and extrudes fibrils of cellulose as part of the metabolism of glucose. The glucose subunits that form the cellulose microfibril are extruded through pores in the cell wall of the bacteria. In standing laboratory culture of the bacteria, the cellulose fibrils bundle together to form a mat or pellicle within which the bacteria are held. The pellicle floats on the surface of the medium allowing the bacteria to obtain plenty of oxygen, which they require for growth, multiplication, and more cellulose synthesis. Cellulose biosynthesis by *A. xylinum* may serve the bacterium in a number of ways in their microenvironment. These may include holding the bacteria at the air interface to ensure adequate oxygen, protection from ultraviolet radiation, protection from desiccation by holding water, acting as a biofilm to hold the organisms on substrates that they may be decomposing, and/or possibly providing protection from predators or competitors.

Cellulose synthesis occurs as a multi-step series of chemical reactions beginning with glucose that is catalyzed by enzymes. The pathway is believed to be as shown in Figure 1. Cellulose is considered to be a secondary metabolite, which the organism synthesizes when major nutritional needs are met. There is genetic and molecular evidence that cellulose synthesis in *A. xylinum* is under operon control.

Some practical applications of bacterial cellulose include: sensitive diaphragms for stereo headphones, thickeners for foods, paint, ink and adhesives. It has also been used as short term protection for burned skin prior to skin grafting. Most commercial applications of cellulose produced by *Acetobacter* have employed static batch culture to produce sheets of cellulose that can be sterilized to kill the bacteria and washed to remove microbial waste product contaminants. One large scale commercial application is the production of bacterial cellulose in a nonaseptic medium of sugar-enriched coconut milk in the Philippines, yielding an edible product called "nata de coco." This food product has become popular in Japan as a high-fiber, low-calorie food.

Recently, Japanese investigators have developed a technique to harvest *Acetobacter* cellulose from shallow culture pans with a windup roller device to pull the cellulose slowly from the surface of the culture medium. High yield commercial production of bacterial cellulose will require agitated (shaking) culture techniques. There has been some limited success in the laboratory studies producing cellulose in this way, but, at present, there are no commercial applications of this process.

Materials

Acetobacter xylinum that secretes cellulose is available from the American Type Culture Collection, which is located at 10801 University Blvd., Manassas, VA 20110-2209, telephone 703-365-2700, http://www.atcc.org (Strain #23769 for this particular report). It is a nonpathogenic bacterium, but careful aseptic techniques should always be practiced when handling it. *Acetobacter xylinum* is cultured aseptically in the laboratory in liquid or solid medium. The medium consists of the following ingredients:

- Glucose - 20 g/l
- Yeast extract - 5 g/l
- Peptone - 5 g/l
- Dibasic sodium phosphate - 2.7 g/l
- Citric acid - 1.15 g/l

When making solid medium, add agar at 15 g/l. The medium should be sterilized. Pre-mixed medium ingredients are not currently available to culture *A. xylinum*. However, the individual ingredients to make the medium are available from major scientific supply companies such as Fisher Scientific, VWR Scientific or Sigma Chemical Company. The pH of the medium should be between 6.6 and 6.8. The optimum temperature for growth of the bacteria is 30° C, but the organisms can grow well at typical room temperatures.

An easy way to observe the production of cellulose by *A. xylinum* is to culture bacteria aseptically in liquid medium in the bottom of petri dishes.

Glucose (glucokinase)→Glucose-6-Phosphate (phosphoglucomutase)→
Glucose-1-Phosphate (UDP-glucose pyrophosphorylase)→
UDP-Glucose (cellulose synthase)→Cellulose

Figure 1. The pathway of cellulose synthesis.

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First sterilize the medium in tubes holding 10 to 15 ml. The sterile medium can be inoculated with bacteria, and then the broth can be poured carefully into the bottom of a sterile, plastic petri dish, which is then covered to maintain sterility. The inoculated dish can be incubated and observed over time to see the production of cellulose, which covers the surface of the liquid medium. The cellulose can be weighed either wet or dry after three to seven days or more.

What do we want students to do in the science classroom, and how can the study of *A. xylinum* encourage learning? One of the things that we hope for our students is that they will learn that science is a process that includes: a testable hypothesis, manipulation of a variable, measurement, verification of results (repetition of the experiment, a number of similar samples), controls, and statistical analysis of data. Most scientists also expect a controlled experiment to lead to new questions/hypotheses and future experiments.

Methods

One of the authors has used experiments with *A. xylinum* to teach science using an inquiry approach. Experiments l[illegible]n with the students receiving a minimal amount of information about how to culture the organism.

Procedure

Introduce small groups of students to *A. xylinum* by distributing one or more 15-ml portions of sterile medium in test tubes. Using sterile pipets and technique, add 1 ml of *A. xylinum* culture to each tube. Students may choose to grow their culture in the original test tubes or to pour the 16 mls of medium/culture into a sterile petri dish. This procedure should take 20 to 30 minutes for a class to complete. Explain that this is a unique microbe that produces cellulose as it approaches ideal growth conditions. It will be their task to find the conditions that produce the most cellulose, and in that search, they will be describing the ideal conditions for the microbe. All of the dishes and tubes should then be incubated at room temperature for approximately one week.

After one week, the students should be able to see the pellicles of cellulose floating in the test tubes and in the petri dishes. It is important for them to record the relative amounts of cellulose formed and where the cellulose is located in the tube or dish. The cultures should be poured through filter paper, which will trap the cellulose; rinsed well with tap water; dried; and weighed. Alternatively, the cellulose could be weighed wet after rinsing and placing on filter paper.

The data in Table 1 represent results from six lab groups for the initial activity.

An important part of this initial investigation is to have students compare their results collaboratively and to speculate about the conditions that produce the most cellulose. What is it about the petri dish that seems to stimulate more growth? Where is the pellicle of cellulose located in the test tubes or dishes? As they discuss these findings in small groups or in the class, they often note that the petri dish has increased surface area. They may speculate that the increased surface area encourages growth for this microbe because it provides more access to oxygen. From this point, students usually choose to incubate their cultures in petri dishes to maximize the production of cellulose.

Students can then begin designing their own experiments. Led by the challenge to produce the most cellulose in order to gather additional clues about the nature of the organism, they identify a factor that may affect the microbe's growth. Students have designed controlled experiments with the following variables: temperature, amount of glucose, various carbon sources (maltose, galactose, lactose, or even alcohol), pH, amount of yeast extract, amount of sodium phosphate, amount of peptone, and even addition of live yeast. Some of these experiments require a good understanding of molarity (particularly the carbon source experiments). The students must also practice aseptic techniques.

Students should look for patterns of cellulose production in the various experiments and present their results to their class in a seminar format.

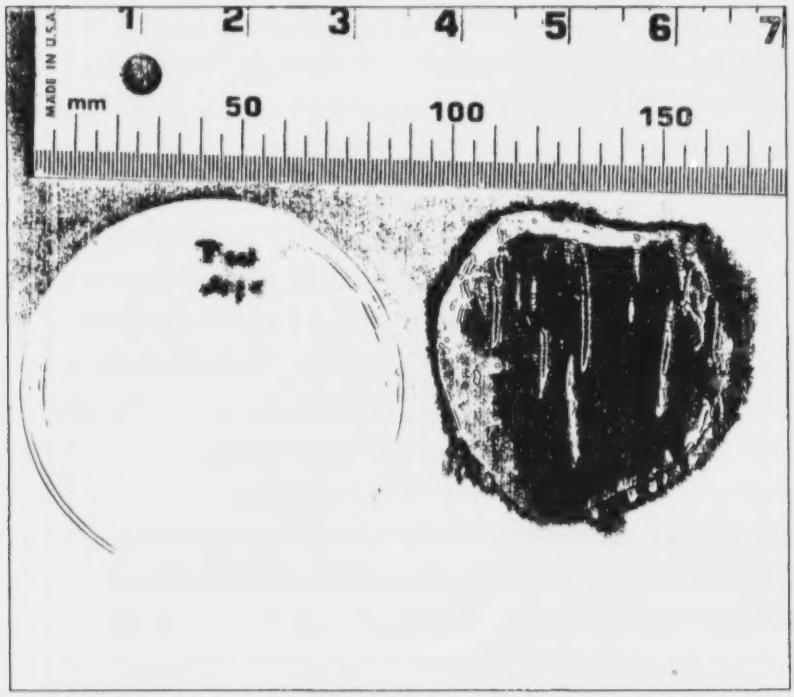

Figure 2. Petri plate (on left) containing pellicle of cellulose formed by *Acetobacter xylinum*. Piece of wet cellulose (on right) produced by *A. xylinum* in a petri plate.

Table 1. Effect of varying surface area on cellulose mass after 1 week incubation.

Surface Area	*Cellulose Mass (after 1 week incubation)*
test tube	0.036 g
test tube	0.089 g
test tube	0.073 g
petri dish	0.668 g
test tube	0.104 g
petri dish	0.492 g

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Table 2. Effects of varying carbon sources and temperature on cellulose production.

Carbon Sources (15 ml of medium + 1 ml culture, 3 day incubation)

Condition	Mass of Cellulose (g)
No Glucose (neg. control)	0.026
Maltose	0.120
Galactose	0.029
Lactose	0.030

Temperature (15 ml of medium + 1 ml culture)

Temperature	Mass of Cellulose (g)
4° C	0.001
22° C	0.052
30° C	0.105
37° C	0.041

The data in Table 2 represent two student groups and their experimental results.

From these results and their preliminary experiments, students could conclude that increased surface area, a temperature around 30° C, and maltose would produce the most cellulose. These results may lead to the development and testing of new hypotheses, and serve as the basis for concluding statements about the organism based upon the collaborative work of the class.

The *National Science Education Standards* list several components to the scientifically literate student as they describe what students who understand science are expected to be able to do. As we work with unusual organisms and expect students to step outside boundaries of a prescribed laboratory exercise, they should be able to describe objects and events, ask questions, construct explanations, test explanations against current scientific knowledge, and communicate ideas to others. This is the type of laboratory exercise that gives students practice with each of those components.

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Investigating Phagocytosis in *Tetrahymena*

An Experimental System for Introductory & Advanced Instruction

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Tetrahymena, a freshwater ciliated protozoan, is in many ways an ideal candidate for the study of cells by students. Readily obtained and cultured, *Tetrahymena* can be used to study ciliary motion, organelle structure and function, cell morphology, cell proliferation, and cell behaviors (Leick & Hellung-Larsen 1992; Wheatley et al. 1994). Because of the ease with which *Tetrahymena* can be grown and manipulated, it is especially suitable for use in inquiry-based labs and student projects.

Phagocytosis in *Tetrahymena* is an especially dramatic cell behavior that students can observe directly with a compound microscope. When hungry *Tetrahymena* encounter food, they use their cilia to sweep it into each cell's oral groove. This process can be visualized by feeding stained yeast cells India ink (Keenan 1984) or *Chlorella* to *Tetrahymena*. Phagocytosis can be quantitated by counting the number of vacuoles that form in a defined time period.

In this paper, I describe how our students use the India ink method, described below, to investigate phagocytosis and vacuole formation in *Tetrahymena* first to learn the basis of microscope use, and then to pursue various experimental projects.

Procedures

Observation of Phagocytosis

Student Instructions

1. *Tetrahymena* grown in 2% proteose peptone for 48 to 72 hours will be provided.
2. Pipette 3 ml of *Tetrahymena* and 3 ml of 1% India ink into a test tube. Mix gently.
3. Using a compound microscope, observe the behaviors of the cells. In particular, pay attention to how the cells swim and eat. Record your observations.
4. Sample cells at 0, 2, 5, 10, 20 and 30 minutes. To sample, pipette 20 microliters of cell + ink suspension into a microfuge tube containing 10 microliters of 3% glutaraldehyde. Exposing cells to glutaraldehyde, a fixative, will kill the cells. Consequently, you can work at your own pace once the cells have been sampled. Also note that the "0 minute" time point refers to the first sample you are able to collect right after the combination of the cells and ink. In real time, it will be less than one minute.
5. Place a drop of sample cells on a slide and, using a compound microscope at 100 to 200X total magnification, count the number of vacuoles containing ink per cell for at least 20 cells for each time point.
6. Prepare a table and graph to display your results.
7. Design an experiment in which you use this assay to investigate some aspect of phagocytosis.

Information for Instructors. This simple procedure is an excellent one for introducing students to the basics of microscope use. *Tetrahymena* are best observed at either 100 of 200X total magnification. Since the vacuoles that form are full of ink, they are easy to identify (Figure 1). In 1% ink, the movement of the cilia can be observed as can the formation of the vacuoles. Moreover, students can also see the movement of the vacuoles inside the cell as new ones form, and if they are sufficiently patient, sometimes they can even see vacuoles "void" their contents into the extracellular space. If you have a video camera that can be attached to your microscope, a permanent record of student work is possible.

Aseptic technique should be used to grow *Tetrahymena*. To culture, inoculate 1 ml into 25 ml of 2% proteose peptone in a 125 ml Erlenmeyer flask (the ratio of medium volume to total flask volume should be 1:5 or lower). Incubation at room temperature, (20° to 22° C) is best. *Tetrahymena* can be purchased from many suppliers; I use Carolina Biological. Be sure to order the "bacteria-free" cultures.

To make 2% proteose peptone, add 2 g proteose peptone (Difco) per 100 ml distilled or deionized water. Autoclave 20 minutes on liquid cycle or slow exhaust. This medium should be stored in the refrigerator and used within two months.

The type of ink you use for this procedure matters a great deal because some inks have detergent in them; these won't work. I use Hunt-Speedball. All dilutions are made in distilled or deionized water.

Examples of Student Projects

Using the basic protocol described above, students have designed and implemented many projects that explored some aspect of phagocytosis. The three projects presented below show examples of questions students asked and answered experimentally.

Does the Concentration of Ink Affect the Rate of Vacuole Formation? In this experiment, *Tetrahymena* were fed 1, 5 or 10% ink. Cells were sampled at 2, 10, 20 and 30 minutes. As shown in Table 1, the rate and degree of phagocytosis were inversely proportional to the concentration of ink. This observation was a surprise to the students who did this experiment and they did additional research to try to explain why the higher concentrations of ink resulted in fewer vacuoles. While they did not determine the specific mechanism that accounted for this phenomenon, they did articulate additional testable questions to further explore this result. For example, they wondered whether the high concentration of particulates in the ink might have damaged the cilia around the oral groove so that material could not be swept in efficiently. To test this they proposed exposing cells to 5 and 10% ink for 10 minutes, and then washing the cells to see if they would now eat 1% ink at the control rate.

Does the Nutritional State of *Tetrahymena* Affect the Rate at Which Food Vacuoles Form? In this project,

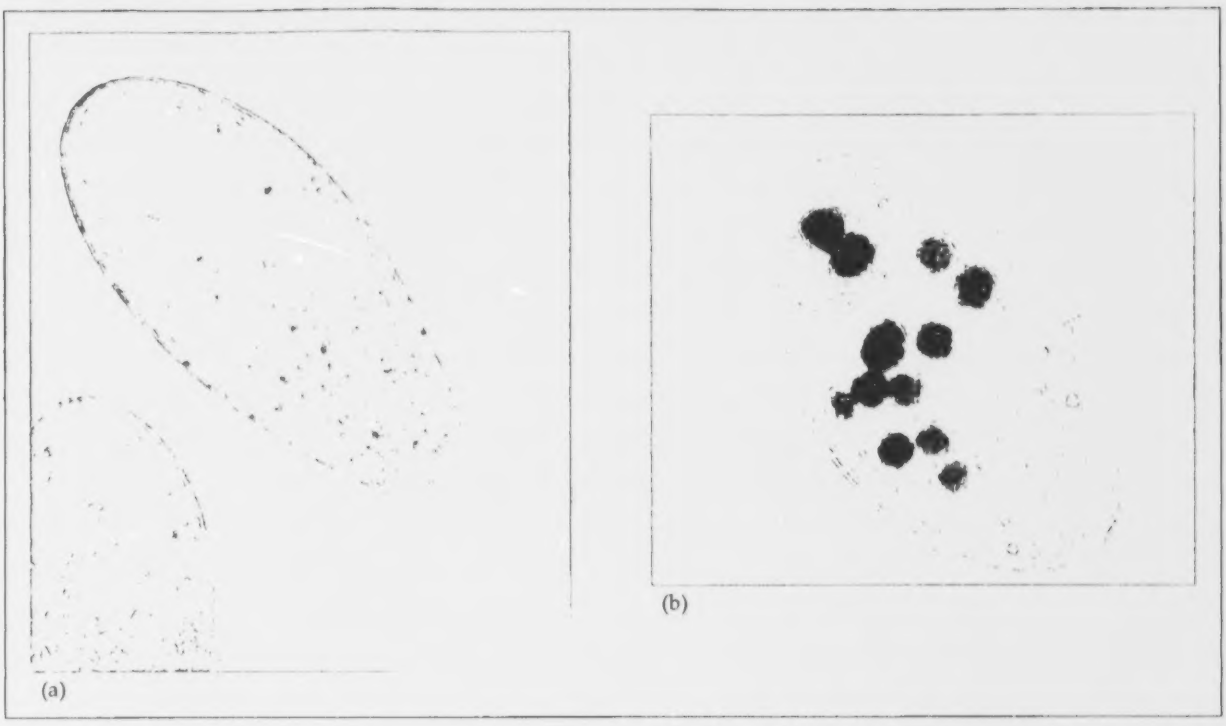

Figure 1. a) *Tetrahymena* fixed in 3% glutaraldehyde. b) *Tetrahymena* fed 1% India ink for 10 minutes and fixed in 3% glutaraldehyde.

Table 1. Phagocytosis of different concentrations of India ink by *Tetrahymena*.

Time (min)	Mean Number of Vacuoles/Cell		
	1% Ink	5% Ink	10% Ink
2	2.6	2.1	0.7
10	3.2	1.7	1.3
20	5.1	2.1	1.3
30	5.2	2	1.1

students wanted to see whether *Tetrahymena* would eat faster if they had been deprived of food for a while. To test this, a well-fed and a starved population of *Tetrahymena* were each fed 1% India ink and sampled at 0, 5, 10 and 20 minutes. The number of vacuoles per cell was determined for each cell sample. As shown in Table 2, the population of cells that were starved exhibited a reduced rate of vacuole formation as compared to the well-fed control. At first, this result seemed counterintuitive to the students but after additional research they reasoned that perhaps starved cells will only eat actual food while well-fed cells will ingest anything that will fit into the oral groove, even ink particles. Again, students were able to design a logical experiment to test this hypothesis: Would starved and well-fed *Tetrahymena* eat *Chlorella*, or yeast, at a similar rate?

Table 2. Phagocytosis in starved and well-fed *Tetrahymena*.

Time (min)	Mean Number of Vacuoles/Cell	
	Well-fed cells	Starved cells
0	0.88	0
5	5.3	1.8
10	6.4	3.1
20	8.5	4.4

In this experiment, the well-fed cells were *Tetrahymena* that had been grown for 48 to 72 hours in 2% proteose peptone. To starve *Tetrahymena*, centrifuge cells from a 48 to 72-hour culture (1000 rpm in a tabletop centrifuge for 4 minutes), and resuspend the pellet in dilute salt solution. Centrifuge and wash the cells two more times. Resuspend the pellet in a volume of dilute salt solution equal to the volume of medium you initially removed. The resuspended cells should be incubated at room temperature. To make the dilute salt solution, add the following to each liter of distilled or deionized water.

- 6 mg KCl
- 4 mg $CaHPO_4$ (or $CaCl_2$)
- 2 mg $MgSO_4$-$7H_2O$

Autoclave 20 minutes at slow exhaust.

Is the Cytoskeleton Involved in the Formation of Vacuoles? Observations of the formation of vacuoles and the movement of those vacuoles within the cell led two groups of students to wonder what role the cytoskeleton plays in this process. To determine whether microfilaments or microtubules were essential for phagocytosis, *Tetrahymena* were exposed to cytochalasin B or colchicine, respectively.

Table 3. Effects of cytochalasin B and colchicine on phagocytosis in *Tetrahymena*.

Time (min)	Mean Number of Vacuoles/Cell		
	1% Ink	Colchicine + 1% Ink	Cytochalasin B + 1% Ink
0	0	0	0.1
10	6.9	3.9	1
20	7.6	5.1	1
30	9.3	5.2	1

More specifically, 2 ml of 48 to 72-hour cultures were incubated in a test tube with either 20 microliters of cytochalasin B or colchicine for 10 minutes. After this incubation, 2 ml of 1% ink were added to each tube. Cells were sampled every 10 minutes for 30 minutes and vacuole formation was quantitated. Table 3 shows the results of this experiment. Cytochalasin B dramatically inhibited vacuole formation. Ink was ingested by the cells, but the vacuole that formed never pinched off and moved into the cell. Colchicine also decreased the rate of vacuole formation. Students hypothesized that its effect was due to the loss of microtubule tracks within the cells. Without these tracks, the vacuoles could not move efficiently into the cell body. Since cytochalasin B is insoluble in water, it is dissolved in a small amount of ethanol (see below). Consequently, we ran a control experiment to verify that cytochalasin B, and not ethanol, was causing the effects observed (Table 4). Additional questions raised by these students include: Are the effects of the drugs reversible? Is the effect of the drugs dose-dependent?

Because this experiment uses cytochalasin B and colchicine, potentially hazardous materials, students should be carefully supervised during this experiment and appropriate precautions should be taken. To prepare a stock solution of cytochalasin B, add 5 mg per ml of 70% ethanol. The final concentration used in the experiment above was 50 micrograms per ml. To prepare a stock solution of colchicine, add 400 mg per ml of distilled or deionized water. The final concentration used was 4 mg per ml. Both stock solutions should be refrigerated.

Additional Possibilities

Using the simple procedure of measuring phagocytosis and vacuole formation by feeding India ink to *Tetrahymena*, students can design and implement experiments of varying complexity. In addition to the explorations described in this paper, examples of other testable questions generated by students include:

- Does the rate of phagocytosis vary with temperature?
- Does the velocity with which *Tetrahymena* are agitated affect the rate of phagocytosis?
- Will *Tetrahymena* select specific items for ingestion when presented with mixtures of food?

Invariably, an experiment done to answer a particular question leads to more questions and ideas for experiments. It is helpful to note that many of the papers that describe basic studies of phagocytosis and vacuole formation are available to provide context for the work that students do (Ricketts 1972; Nilsson et al. 1973; Hoffman et al. 1974). Also, phagocytosis in *Tetrahymena* has, in recent years, become a model for learning about pharmacology and cell membrane function (Stefanidou et al. 1990; Csaba & Darvas 1992; Renaud et al. 1995). Consequently, the projects students do have a connection to current research problems. The ease with which the procedure can be done along with the vast array of potential testable questions that can be pursued make studying phagocytosis in *Tetrahymena* an experimental system rich with possibilities.

Table 4. Control experiment demonstrating phagocytosis in the presence of ethanol, the solvent used in the cytochalasin B experiment.

Time (min)	Mean Number of Vacuoles/Cell	
	1% Ink	Ethanol + 1% Ink
0	1.2	0.7
10	6.7	6.3
20	8.0	8.5
30	8.5	8.8

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Genetics & Biotechnology

Blood Type Compatibility

A Simulation of Medical Transfusion Reactions

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With the prevalence of serious blood-borne transmitted diseases—most notably transmission of the AIDS virus—the use of human blood in laboratory activities has been either severely restricted through rigid protocol recommendations (Vetter 1989), or has been completely proscribed in many school districts throughout the country. To counter this restriction, alternative activities have been developed including kits and computer software simulating blood typing, prepared slides with which students can observe blood components, and audio-visual aids diagramming blood processes. Sharp and Smailes (1989) outlined a sophisticated procedure to simulate blood type testing using mixtures of inorganic solutions in the April, 1989 issue of *The American Biology Teacher*.

I have discovered a simple, inexpensive, easily-prepared demonstration to be used to teach the related concepts of human blood grouping, antigen-antibody reactions, and medical transfusions in a stimulating way. The method is equally adaptable to biology/life science courses at the introductory and advanced levels.

The Role of Blood Groups in Transfusions

Since the historical discovery of human blood groups and their specific badge of glycoprotein antigens on the surface of erythrocytes by Karl Landsteiner in 1900, medical transfusions have been carried out safely without fear of patient rejection. Prior to this, life-threatening reactions by recipients to incompatible blood donor types were not understood. In light of Dr. Landsteiner's discovery, this rejection is a direct result of antibody action against an opposing blood type antigen causing severe agglutination (clumping) of the erythrocytes. This tends to block blood vessels, resulting in brain and kidney damage and, ultimately, death (Tortora 1990). Antigenic/antibody components of specific human blood types and their transfusion relationships may be obtained in most introductory biology texts (Keeton & Gould 1986).

Although blood type "O" contains plasma antibodies against blood types "A" and "B", it can sometimes be used in emergency transfusions as a donor to these blood types since the antibodies in this blood type become so diluted in the recipient's plasma upon administration that the amount of agglutination does not reach lethal levels. Nevertheless, a sincere attempt to cross-match blood types exactly is made whenever possible.

The process of medical transfusions is of great interest to students and has versatility in discussing related concepts of antigen-antibody reactions, circulatory system physiology, and the need for blood during surgery or after trauma—as when a dangerous decrease in blood volume has occurred.

Discussion on the relevance of transfusions to medical procedures may also lean toward their life-saving potential in saving unborn babies with blood types that are incompatible to those of their mothers—a serious disease known as erythroblastosis fetalis. Or the discussion may revolve around challenging medical consequences associated with transfusions as in the transmission of the HIV virus to recipient hemophiliacs. In addition, by researching current procedures used to screen blood that has been donated or methods for storing blood autologously, the students will remain abreast of developments in this area. The simulation demonstration that I propose will enable the students to visually appreciate the significance of protein antibodies in antigen-antibody reactions associated with the immune system. In addition, the consequent concept of correctly matching blood types, based on preventing these reactions during true medical transfusions, will also be obtained.

Materials & Methods

Supplies

The following materials and equipment are required to simulate your own classroom "transfusions":

- Skim milk (not whole or low-fat)
- White vinegar
- Five 250-ml flasks with stoppers
- Four petri plate lid bottoms (preferably glass)
- One 10-ml volumetric pipet (or 10-cc hypodermic syringe, if available)
- Red food coloring
- An overhead projector.

Label the undersides of each of the four petri plate bottoms with the symbols "A", "B", "AB" and "O" in the center of each plate. Use a waterproof permanent marker and write the symbols backwards in such a way that they will appear in normal position on the overhead projector screen when the lids are placed in the filling position on the overhead stage.

Preparation

Prepare the flask solutions ("blood types") used in demonstration according to the schedule below (each in a 250-ml flask):

Type A:	200 ml of 5% skim milk (10 ml milk diluted with water to a volume of 200 ml); keep refrigerated when not in use
Type B:	200 ml white vinegar
Type AB:	200 ml water
*Type O_I:	200 ml water
*Type O_{II}:	200 ml white vinegar

*These solutions would be used to portray different simulation events by "O" types for demonstration only and do not imply a subdivision of the basic blood type.

Add approximately 20 drops of red food coloring into each flask to give the appearance of blood. After preparation, these flasks and their markings should be concealed from view to prevent confusion by the students.

Procedure for Demonstration

A gradual clumping of milk particles by the vinegar is the basis for evidence that "agglutination" of incompatible types during transfusion has occurred. The acetic acid in the vinegar

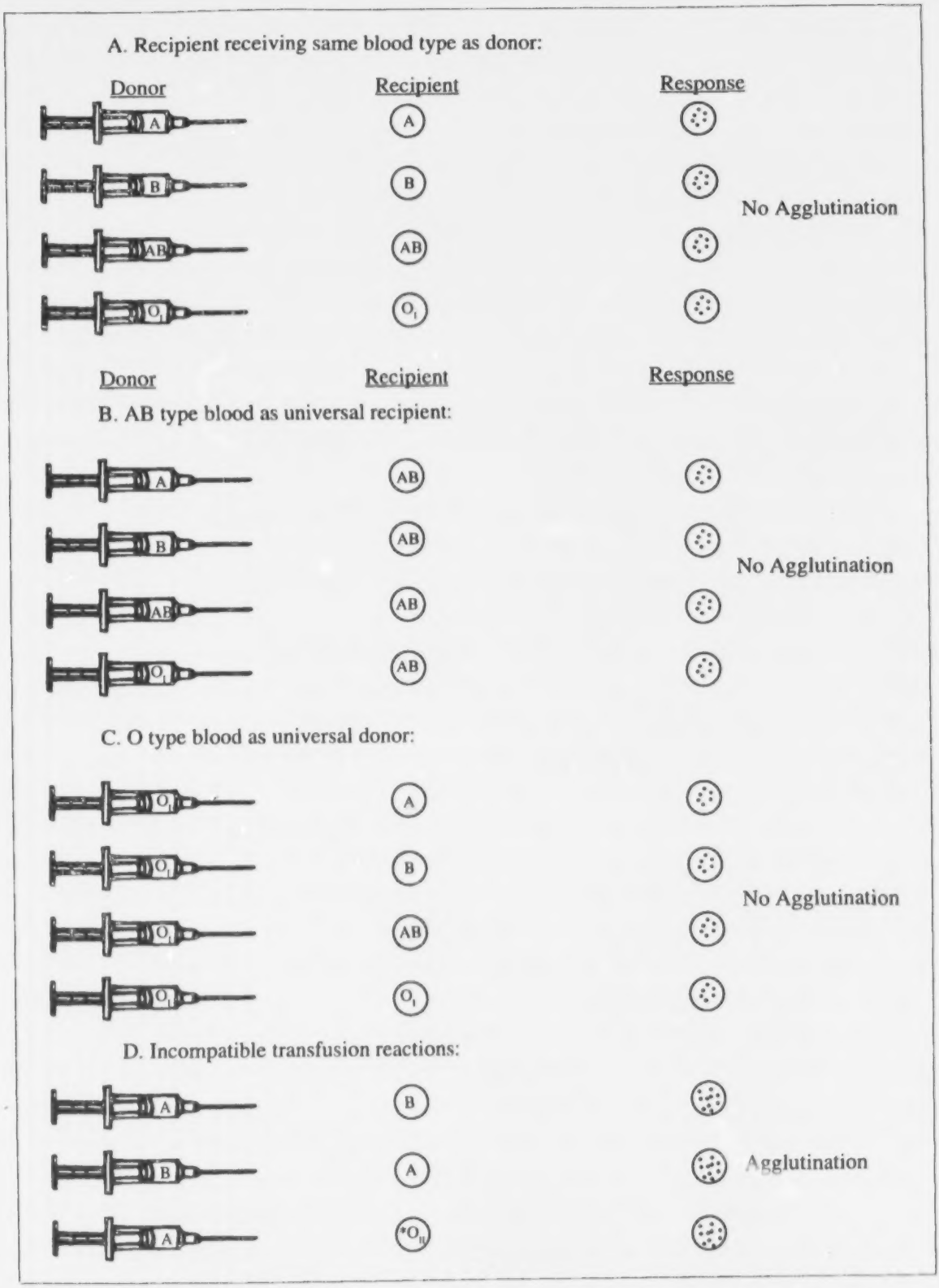

Figure 1. Possible combinations of donor vs. recipient transfusion fluids using the above protocol and their outcomes.

chemically precipitates milk protein (casein) into partially insoluble curdles. This clumping—along with darkening of the reaction mixture—is easily observable when the petri plates that have been administered an incompatible "blood type" are placed on the overhead projector. Students generally show a heightened anticipation of the results whenever a test is performed.

After preliminary classroom discussion on the role of blood types, antigens, antibodies, and mode of action, along with an understanding of the opportunities for transfusion requirement, questions are posed to the class in such a way as to predict whether or not a "transfusion" will be compatible in each case. For example, the demonstration may begin by asking the fundamental question of whether or not an individual can safely receive his/her own blood type during a transfusion. The possibility can be entertained by placing petri lid "A" (or any other type) representing the "recipient" on the overhead projector stage and just covering the bottom of the lid with type "A" flask solution (from preparation schedule above). After soliciting a prediction of the outcome by the class, a 3- to 5-ml sample of type "A" (the "donor") is slowly pipeted into the solution in the lid. After waiting approximately 1–2 minutes for a response, it will become evident on the overhead projector screen that no clumping or darkening of the mixture will have taken place, thereby representing a "safe" transfusion.

To show the universal donor status of type "O" blood, use solution "O_t" as the donor and pipet into plates carrying types "A", "B", "AB" or "O_t" as recipients. In like fashion, you may demonstrate the universal recipiency of "AB" blood by using the "AB" plate/solution as recipient and pipeting into it at different times or simultaneously types "A" (*or* "B"), "AB" or "O_t". Neither will invoke a reaction. [Note: Do not pipet types "A" or "B" together into the same lid at any time unless clumping is desired; any mixture involving the milk and vinegar together will cause "agglutination."]

To demonstrate an incompatible transfusion such as type "A" to "B" or vice-versa, place a lid on the stage with type "B" just covering the bottom of the "B" lid. Have students predict beforehand what will occur if we transfuse with "A" type blood and why. Then *slowly* add 3–5 ml of this type to the lid and watch the screen. Within 1-2 minutes easily discernible "agglutination" clumps and darkening will be visible. Discuss the results. For heightened drama in each case, I use a 10-cc hypodermic syringe complete with matching needle obtained from a medical supply store in lieu of a volumetric pipet. The donor blood is slowly aspirated into the syringe from the appropriate flask and then carefully "injected" into the recipient fluid in the receiving lid. For obvious safety reasons, students are not allowed to use the syringe.

Figure 1 lists all of the possible transfusions that can be demonstrated to your students with this technique and their outcomes.

During or after the discussion, the topic may then revolve around the function of antibodies and how they may, once encountering a specific antigen, such as a blood antigen, initiate an antigen-antibody reaction on the donated blood. In the case of incompatible blood transfusions, the clumping is actually a precipitation response due to cross-linking of the Y-shaped antibodies with the antigen protein molecules on the red blood cell surface resulting in an immobile precipitate (Campbell 1993). The effect is similar to the center piece of a jigsaw puzzle tightly binding the surrounding pieces into a permanent, immovable unit. It may also be noted during observation of agglutination how this insoluble clumping would affect the tissues and organs of the body as a whole. For example, it should be mentioned to the students that brain neurons and heart muscle fibers require a constant, prodigious amount of oxygen in order for them to function effectively in their respective organs. Immobilized clumps of blood would inhibit the diffusion of this essential gas from the red blood cells to these tissues and would be responsible for their eventual shutdown. In more advanced classes, a more in-depth discussion of this effect could concentrate on how a lack of oxygen would prevent acceptance of H^+ ions at the end of the electron transport (cytochrome) chain along the mitochondrial cristae. This would result, of course, in a reduction in regeneration of ATP from ADP in oxidative phosphorylation, thereby producing a severe deficit in energy availability to the tissues.

Thus, depending on the degree of explanation desired, a greater understanding of how small, invisible reactions may result in more magnified effects at the body level. Moreover, this activity can be used to cultivate the higher cognitive skills of prediction and application, important components in any science classroom.

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Introducing Mendelian Genetics Through a Learning Cycle

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A primary goal of biology instruction is to engage students in investigations that are real to them so they learn specific biological concepts and theories as well as develop an understanding of the nature of scientific processes and develop skill in using those processes (e.g. American Association for the Advancement of Science 1989; National Research Council 1990). One instructional method that has been found to be successful at engaging students in meaningful inquiries is the learning cycle. Learning cycles consist of three instructional phases: exploration, term introduction, and concept application (Atkin & Karplus 1962; Karplus & Thier 1967; Lawson 1988, 1991; Lawson, Abraham & Renner 1989; Science Curriculum Improvement Study 1974). During exploration, students investigate new phenomena that may raise questions and may enable them to discover patterns in those new phenomena. Term introduction allows teachers to introduce biological terms related to the discovered patterns, while concept application activities enable students to attempt to apply newly constructed concepts to new contexts to broaden and deepen their understanding.

Student perceptions of differences between learning cycle and traditional verification labs were identified by Abraham (1982). Using a Q-sort instrument, chemistry students ranked 25 statements according to how accurately they characterized their lab activities. The following statements were ranked significantly higher by the verification lab group:

- The instructor is concerned with correctness of the data.
- The instructor lectures to the whole class.
- During the lab, students record information as requested by the instructor.
- Experiments help students develop skill in the techniques or procedures of chemistry.
- Students usually know the general outcome before doing the experiment.

The following statements were ranked significantly higher by the learning cycle lab group:

- Students are asked to design their own experiments.
- The instructor requires students to explain why certain things happen.
- Lab reports require students to use evidence to back up their conclusions.
- Students propose their own explanations for observed phenomena.

Thus the verification labs were characterized by correctness, lecture and the development of lab techniques, while the learning cycle labs were characterized by experimentation, explanation, observation and the use of evidence.

Effectiveness of the learning cycle method has been assessed in several studies since it was first proposed in the late 1960s in several contents and at virtually all educational levels. Space does not permit a review of these studies here, but a review of more than 60 such studies can be found in Lawson, Abraham and Renner (1989). The Lawson, Abraham and Renner review concluded that the learning cycle method is very effective in terms of improving students' attitudes, achievement and scientific reasoning skills. Perhaps one of the most important of these studies was conducted by Renner et al. (1973). In the study, elementary science students who had used the learning cycle activities contained in the Science Curriculum Improvement Study (SCIS) program for at least four years were compared to students who had been taught textbook science for the same length of time. SCIS students were found to have greater skill in using the processes of observing, classifying, measuring, experimenting, interpreting and predicting. The study then looked at the possible transfer of these skills to other areas of the curriculum. Scores on the Stanford Achievement Test administered during the fifth grade showed the SCIS students' scores to be significantly better than those of control students in mathematics applications, paragraph meaning and social studies skills—all areas that require the application of reasoning skills. In another important study, Brown, Weber and Renner (1975) found no significant difference between the attitudes of the SCIS students and those of professional scientists.

The learning cycle has many advantages over traditional instructional approaches, especially when the development of thinking skills is an important goal. Because many studies have shown that a large proportion of the secondary and college population have poorly developed thinking skills, it seems reasonable to conclude that the learning cycle deserves more widespread implementation in science classrooms.

How can the learning cycle method be used to provoke student inquiry into concepts of Mendelian genetics? A recently developed learning cycle designed to do just that starts by posing the descriptive question, "How do characteristics vary within species?" Students intuitively know that all members of a species share many characteristics, but all members are not identical. Variations in characteristics exist, but is there a pattern to that variation? And if so, what is it? To answer these questions, students explore the characteristics of a variety of mollusks (sea shells) and color frequencies of kernels on ears of Indian corn. This exploration allows students to discover a pattern of variation in which few shells are found at the extremes of the range in variation but many are found in the center of the range. The discovery allows the teacher to introduce the term *normal variation* and to raise the causal question, "What might be happening in nature to cause this pattern of normal variation?" The 1:2:1 pattern of kernel color variation on some ears of Indian corn looks somewhat normal, but the 2:1 and 1:1 ratios of others are not at all normal. These conflicting observations raise the corresponding question, "What might be causing these patterns?" Students generate alternative hypotheses to try to answer these questions and then try to put some of their hypotheses to an initial test. Along the way, the teacher has

an opportunity to introduce several principles of Mendelian genetics, and the students have opportunities to try to apply them to explain their observations.

Details of this learning cycle will be presented in two sections. The first section, titled *Student Materials*, includes an introduction, a materials list, a procedure to guide the student inquiry, and a set of application questions to extend the lesson. The second section, *Teacher Materials*, includes content-related background and teaching tips for each phase of the learning cycle. The lesson is one of a collection of 38 learning cycles developed for use in introductory biology classes (Lawson 1994).

Student Material

How Do Characteristics Vary Within Species?

When organisms in a particular environment share many characteristics, interbreed in nature, and produce fertile offspring, they are said to be all of one kind—one species. But how similar are all the members of a single species? Obviously they are not all identical. Humans are all one species yet differences among us in skin color, eye color, height, weight, and so on are obvious. Or, for example, suppose you find two trees that appear the same except one has leaves that average 5 cm across and the other has leaves that average 3 cm across. Are the trees still of the same species? In what ways can individuals of a single species differ? Are these differences systematic or random? What are possible causes of these differences?

Objectives

1. To investigate and discover patterns of variation within a variety of species.
2. To learn to graph data that organize and summarize within species variation.
3. To propose and initially test alternative hypotheses to explain the observed patterns of variation.

Materials

assorted shells
pairs of dice
coins
metric rulers
ears of Indian corn

Procedure

1. The first question we would like to address is this: *What is the pattern(s) of variation of characteristics within species*? In order to answer this question select a lab partner and obtain a large sample of several different kinds of shells.
2. Sort the shells into groups that you think may represent separate species. List characteristics used for your sorting.
3. How many species do you think are present? Explain.
4. Select one species that exhibits differences that can be observed and/or measured.
5. Collect as many individuals of that species as you can throughout the lab. If possible, your sample should contain at least 100 specimens.
6. For each specimen, determine the value of the characteristics chosen. Record the frequency of occurrence of the values in the table below.
7. What characteristic did you measure?
8. Plot your data on a frequency graph. Title the graph and label both axes.
9. What is the average, median, mode and range of your data? (Note: Your instructor may want you to skip this step until you have completed Questions 10–15.)
10. The graphs for each species will be collected, put on the board, compared, and contrasted. The graphs should enable us to answer the question, *What is the pattern(s) of variation of characteristics within species*? and should raise additional questions such as *What causes the observed pattern(s) of variation*? To help answer this question do the next activity with the dice.
11. Take two die and roll them. Note the numbers that turn up on each dice. Add these numbers and record the sum. Repeat this 100 times and plot the data on a frequency graph. Compare this graph with the previous graphs. Do the patterns suggest a mechanism for the observed variation within species? Explain.
12. Obtain an ear of Indian corn. Count the number of kernels of each color on the ear. How many kernels of each color were there? Plot these data on a frequency graph. Approximately what ratio of colors did you find?
13. Take two coins and flip them 100 times. Note the number of times a flip results in one head and one tail, two heads, two tails. Record these numbers and plot them on a frequency graph.
14. Compare your graphs with your previous graphs. How are they similar, different?
15. Compare your results to those of other students. Do these data suggest a cause for the observed patterns of variation? Explain.

Table 1. Frequency of occurrence of values.

Values	*Frequency*

Application Questions

1. What is the difference between a sample and a population?
2. How could you obtain a representative (unbiased) sample of the population of students at your school?
3. Genotype refers to the collection of genes in an individual's body cells. Each body cell has the same collection of genes—the same genotype. Sex cells, or gametes, have only ½ of the genes of a body cell. Given the following genotypes, how many different kinds of gametes are possible? Assume that in this case the genes assort themselves independently of one another (i.e. there is no linkage).

Parental Genotypes:

a. Dd b. DdEe c. DdEeff

4. What is the probability of getting the gamete de from each of the following genotypes?
   a. DDEe b. Ddee c. ddee
5. What is the probability of each of the following sets of parents producing the given genotypes in their offspring?

Parents	*Offspring Genotype*
a. DD × Dd	Dd
b. Dd × Dd	Dd
c. DdEe × DdEE	DdEe
d. DdEe × Dd Ee	DDEE

6. In cats, the gene for lack of color is recessive to the gene for normal coloration. If two heterozygous (means they have one of each kind of gene) cats have kittens,
   a. and a kitten has normal coloration, what is the probability that it is a carrier of the recessive gene?
   b. What is the probability that a kitten will lack color?
7. In certain bean plants, the gene L for large pods is dominant over the gene for short pods (l).
   a. If both individuals are heterozygous, what will be the genotypic and phenotypic ratios of the offspring?
   b. If a homozygous (i.e. both genes are the same) long and a homozygous short are crossed, what will be the genotype and phenotype of the offspring?
8. Eye color of the imaginary Grizzly Gronk population of the White Mountains varies. Some Gronks have purple eyes, some have white eyes, and some have orange eyes. Professor Greengenes has discovered that whenever two purple-eyed Gronks mate, they always produce purple-eyed offspring. Likewise, whenever two orange-eyed Gronks mate, they always produce orange-eyed Gronks. But when white-eyed Gronks mate, they are able to produce offspring with three colors of eyes.
   a. Use Mendelian theory to explain how eye color is determined among Gronks (i.e. what is the genotype of the white-eyed Gronks and how can they mate to reproduce offspring of all three colors of eyes?).
   b. Use your theory to predict the phenotypic ratio of offspring if purple-eyed and white-eyed Gronks were mated.
9. Assume that beak color in a population of birds is determined by one pair of genes that produces phenotype color variation from brown to white. A single allele (B), which makes up 50% of the alleles in the population, produces brown pigment such that a bird with the BB genotype will have a dark beak. A Bb bird will have a tan beak and a bb bird will have a white beak (i.e. no pigment is produced). Given a randomly mating population of birds, what phenotypic ratio of beak colors would you expect in the population? Show your work.
10. What form would the frequency distribution of shades of beak color look like if beak color were determined by the additive effect of six or more gene pairs? (No calculation needed.) Why?

Teacher Material

How Do Characteristics Vary Within Species?

Background Information for the Teacher

Observable characteristics are the product of an organism's genetics and environment. When continuous varying population characteristics such as height or weight of individuals are plotted on a frequency graph, the values often distribute themselves in a "bell-shaped" or normal distribution. This pattern can be partially explained by assuming that the characteristic under consideration is determined by multiple pairs of genes that are passed from parents to the offspring with each gene (allele) independently combining with others in a random fashion, much like the sum of the numbers on two dice rolled on a table.

Observed patterns of either-or characteristics such as sex, handedness, tongue-rolling ability, or kernel color on ears of Indian corn can be explained by assuming that the offspring characteristic is determined by a single pair of genes (or very few genes) that somehow combine their effects in the offspring (blending inheritance) or, in some cases, one gene of the pair may dominate the other to produce its effect as though the other gene were not present. Combinations of dominant and recessive gene pairs result in simple ratios of offspring characteristics (e.g. 1:1, 3:1, 1:0) that depend upon the precise combination of dominant and recessive genes.

Gregor Mendel is normally credited with inventing these concepts of inheritance. They can be summarized by the following eight postulates of classical genetics theory:

1. Inherited characteristics are determined by particles called *genes*.
2. Genes are passed from parent to offspring in cells called *gametes* (egg and sperm).
3. An individual has at least one pair of genes for each characteristic in each cell except in the gametes.
4. Sometimes one gene of a pair (an allele) masks the expression of the second allele (*dominant/recessive*).
5. During gamete formation, paired genes separate. A gamete receives one gene of each pair.
6. There is an equal probability that a gamete will receive either one of the genes of a pair.
7. When considering two pairs of genes, the genes of each pair *assort independently* to the gametes.
8. Gene pairs separated during gamete formation *recombine randomly* during fertilization.

You may wish to provide these eight postulates to your students at the conclusion of the term introduction phase as a summary of the major ideas. Do *not* introduce them prior to that time as doing so would restrict student thinking and detract from the naturally motivating exploratory nature of the activity.

Advance Preparation

Assorted shells are available from Delta Education (P.O. Box 915, Hudson, NH 03051-0915, (800) 258-1302, FAX (603) 880-6520). Indian corn is available from Carolina Biological Supply Company (Gladstone, OR 97027, (800) 547-1733, FAX (503) 656-4208, or 2700 York Rd., Burlington, NC 27215, (800) 334-5551, FAX (919) 584-3399). Ears of corn with 3:1, 1:1 and 1:2:1 ratios of colored kernels should be purchased. Remove the labels before the ears are given to the students.

Teaching Tips

Exploration

1. Use the introduction from the student material as a basis for

introducing the lesson. Then have each group of two or three students obtain a bag of 300–400 shells of 8–10 species.

2. Although students may need suggestions on what sort of shell characteristic to measure (e.g. length, width, # of ribs, shades of color), encourage groups to select different characteristics. You may need to go over definitions and methods of calculating or identifying the mean, median, mode and range of their distribution. Or you may decide to omit this step or assign it later, perhaps as a homework assignment.

Term Introduction

3. After the graphs have been drawn, have one member of each group tape their graph to the board so that all the graphs can be seen and discussed. Ask the students to try to state how the graphs are similar/different. Is there a similar pattern displayed by most of the graphs? The students should note that most graphs show that there are few shells with values at the extremes of the ranges but many near the middle. When this pattern is noted, tell students that this pattern is referred to as a *normal distribution* because it is what normally occurs when the values of continuous variables such as the ones measured here are plotted. Write the phrase *normal distribution* on the board. Ask them to think of other characteristics that might show normal distributions when similarly graphed.
4. Now pose the causal question, "What might be occurring in nature to cause values to be distributed normally?" Allow students to suggest alternative explanations. Do not hesitate to call on students who have not raised their hands. In fact, when attempting to elicit alternative hypotheses, it is best to call on students at random to elicit a broad range of ideas. Treat the process of alternative hypothesis generation as a brainstorming session in which all ideas are initially accepted without critique from you or from other students. This practice encourages student participation. The testing of ideas should come only after all the possibilities have been generated. Alternative hypotheses should be listed on the board. Ideas will typically center around environmental effects, e.g. the big shells got eaten and the little shells got smashed by waves so the middle-size shells survived best. A student may suggest a genetic cause but most likely will not be able to explain how genetics could lead to a normal distribution. This is OK because the dice activity will be used shortly to help explicate the process.
5. Graphs that do not show normal distributions should also be discussed. Have the students speculate on why these non-normal patterns emerged. Do not press for agreed upon explanations at this point. Go on to Procedure Step 11 and ask students to think about how the dice-rolling activity might be related to the shell distributions.
6. After the dice-rolling activity is complete, have one graph from each group placed on the board. In a class discussion compare graphs and note the similarity in pattern. Again, a normal distribution will be seen. Ask again why the dice rolling seems to be related to the shell situation.
7. Go on to Procedure Steps 12–13. Again compare data and again ask students how the corn, dice and shell situations appear similar/different.
8. To introduce the general postulates and terms involved in classical gene theory to help explain the observed data patterns, ask students to imagine that one dice is analogous to the female egg and the other to the male sperm. Imagine that the numbers on the dice represent factors or *genes* that somehow dictate observable characteristics in offspring—the values of those characteristics being determined by the sum of the numbers shown in the combined egg and sperm. If one imagines that six possible "types" of sperm and six possible "types" of eggs (i.e. 1, 2, 3, 4, 5, 6) exist, then there are 36 total combinations of sperm and egg types: 1 combination totaling 2 (1 + 1 = 2); 2 combinations totaling 3 (1 + 2 = 3; 2 + 1 = 3); 3 combinations totaling 4 (1 + 3 = 4, 2 + 2 = 4, 3 + 1 = 4); 4 combinations of 5; etc. It helps if you draw the six hypothesized sperms on the board and the six hypothesized eggs. Use the arrows from the sperms to the eggs to represent possible combinations. Plot these combinations on a frequency graph to show students that a normal distribution results, as was the case for the shells. Thus, if we assume multiple genes exist and behave as the dice, then we have a theory to account for the observed normal distributions.
9. We now have a theory that appears to explain why we found the normal distributions for the continuously varying characteristics. But how can the 3:1, 1:2:1 and 1:1 corn kernel distributions be explained? Do we need a new theory or can the present theory be somehow modified to work? If you pose this question and stand back and let your students ponder for awhile, some student (one who most likely has heard the terms dominance and recessive before) will invariably come up with the idea that the corn may have fewer genes and that one may be dominating the expression of the other(s). When this happy idea strikes, you have an opportunity to explicate the process and introduce the terms *dominance, recessive* and *blending inheritance* to the entire class. The coin toss activity can be used as an analogue, much like the dice activity was used previously. The terms *allele, genotype* and *phenotype* can also be introduced.
10. You should emphasize the theoretical nature of the principles introduced in the sense that we really have not observed genes behaving in these ways and controlling various characteristics. Therefore, we do not have direct evidence that genes exist. But we can imagine that they do exist and we can indirectly argue that *if* they do exist, and *if* they behave as claimed, *then* specific patterns of characteristics should be observable (e.g. normal distributions, 3:1, and 1:2:1 ratios). Because these patterns were observed, we *therefore* have at least initial indirect evidence to support the theory. Making an argument of this if . . . then . . . therefore form will help some of your students begin to understand the hypothetico-deductive nature of modern science.

Concept Application

11. For concept application, patterns of inheritance should be discussed in other organisms (e.g. pea plants, chickens, cattle) and the application questions that appear at the end of the investigation should be assigned. Subsequent learning cycles may also enable students to apply some of the concepts introduced here (Lawson 1994).

Biological Terms
- normal distribution
- genes/alleles
- independent assortment
- random recombination
- genotype
- phenotype
- dominant
- recessive
- blending inheritance

Thinking Skills
- accurately describe nature
- state causal questions
- create alternative hypotheses
- generate logical predictions
- organize and analyze data
- draw and apply conclusions

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Rapid DNA Fingerprinting Lab With Unknowns

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The mini-gel method for DNA electrophoresis, popularized for high school use by Cold Spring Harbor Laboratory (Micldos & Freyer 1990) and incorporated in most commercially available kits, takes too long to be performed during a school period of 45–55 minutes. In designing biotechnology labs for the High School Outreach Program of the Center for Molecular Biotechnology at the California Institute of Technology, we modified popular lab methods to allow students to complete the electrophoresis and staining of DNA samples within one 45-minute period. Observing the gel and making a permanent record of its patterns take place on a subsequent day. Our lab saves time by using thin gels that:

1. Solidify quickly
2. Tolerate relatively high current without melting
3. Permit rapid penetration of stain.

Before starting the lab, students spend a period practicing micropipeting, pouring gels, and loading wells.

Typical DNA restriction analysis labs are confirmation activities, in which every lab station receives samples of the same DNA, and the production of expected results shows that the students "did it right." We designed our lab to use several DNA unknowns, so students can gain experience in observing and comparing the resulting patterns. Each student team is given a sample of DNA that is described as having been found at the scene of a crime. Advanced students perform digests with two different restriction endonucleases and compare their electrophoresis results with provided patterns for "suspects." Basic students are given samples of predigested DNA.

The Material

Each lab station is equipped with a four-toothed comb, a gel box containing tris-acetate electrophoresis buffer (Maniatis et al. 1982), a fine-tipped waterproof marking pen, a 1.5-ml tube of loading dye, and a 2–20 µl micropipetor with tips. Two lab stations share one 250 volt, 500 milliampere power supply.

At the beginning of the period, each team of two to four students goes to a central location in the lab and picks up a plastic bag containing one 50- × 75-mm glass slide, one 5½-inch square disposable polystyrene weighing tray, and one 1.5-ml test tube for each student. Other supplies at the central location include stocks of DNA unknowns, 1.2% melted agarose (kept liquid in 250-ml flasks in a water-filled electric frying pan set to maintain a temperature of about 65° C), disposable 10 ml pipets and pipet bulbs for dispensing the agarose, 0.025% methylene blue stain, and overhead transparency film cut into pieces approximately 80 × 120 mm.

The Analysis

The students at each lab station set the glass slide on the table top and dispense 7 ml of melted agarose onto the slide from a disposable 10-ml pipet. Surface tension prevents the agarose from running off the edge of the slide. Students set the comb into the liquid agarose about one centimeter from one end of the slide. The agarose solidifies in less than 5 minutes, during which time each student on a team uses the micropipet to transfer an 8-µl DNA sample (described as having been found at a crime scene) into his or her 1.5-ml tube. Each student then adds 2 µl of loading dye and mixes well.

After about five minutes, the comb is gently removed from the solidified agarose gel. The gel, still supported by the glass slide, is loaded onto the platform of the gel box. The buffer should barely cover the gel with no dimples at the wells. The 10-µl samples are loaded into the wells in the gel. The gel box is closed and the power supply switched on. Gels are run just under 200 volts for 15 minutes. Typical current readings are 50–100 milliamperes per gel box.

At the conclusion of the run, the gel (still supported by the slide) is removed from the gel box and gently slid off the slide into the disposable weighing tray. Methylene blue stain is added barely to submerge the gel, and the gel is rocked gently for four minutes. The stain is poured off, and the gel is rinsed in deionized water and set aside to destain overnight. Student teams discard used sample tubes and turn in the used glass slide, readying the lab for the next period.

During a subsequent period each student sets a piece of overhead transparency film directly on the gel and uses a waterproof marker to trace the outline of the gel and his or her pattern of DNA bands to make a permanent

Figure 1. Electrophoresis patterns obtained with predigested DNA. Stained with methylene blue. Suspect D: pBR322 digested with BstN I. Suspect E: Phi-X174 DNA digested with Hae III. Suspect F: 100 bp DNA ladder. Victim: 1 Kb DNA ladder.

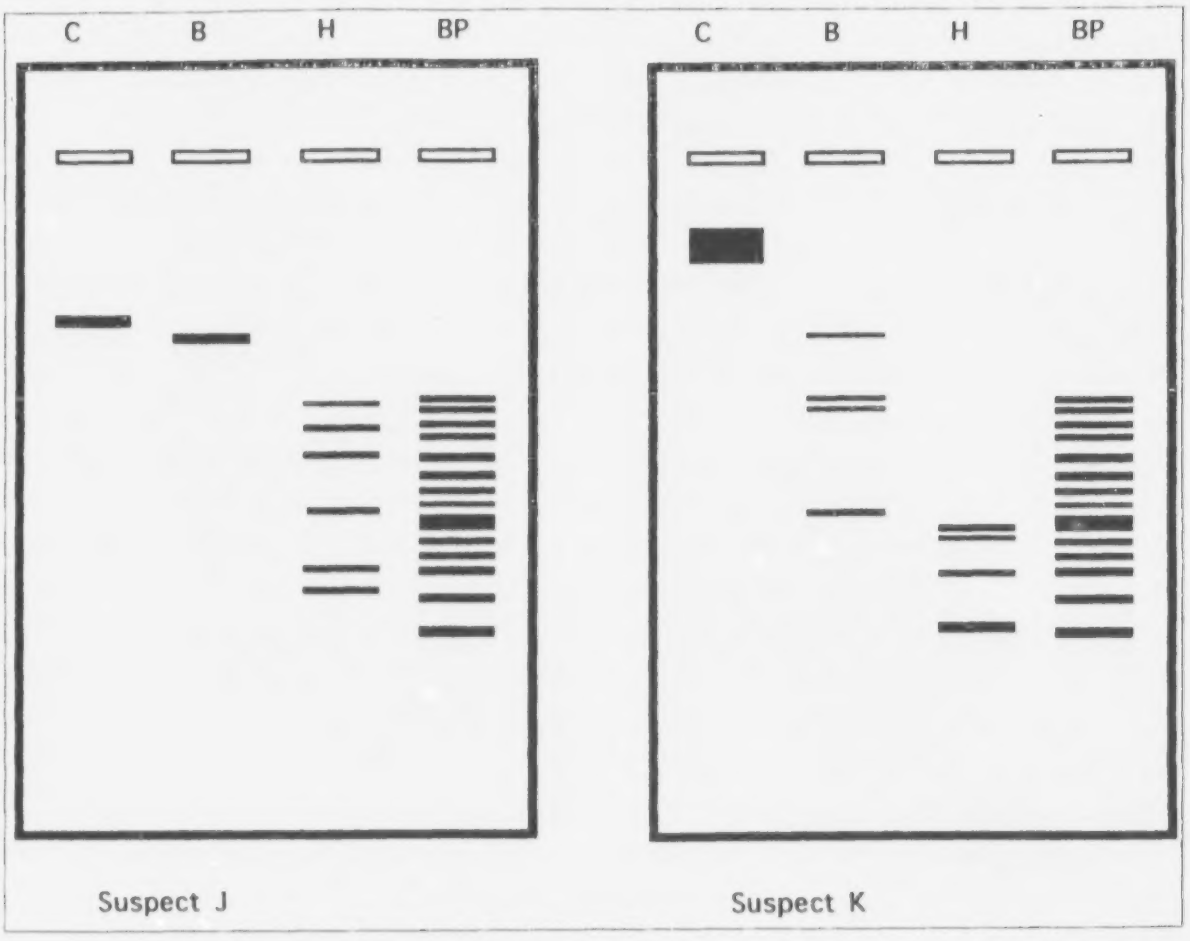

Figure 2. Electrophoresis patterns obtained with DNA digested with two restriction enzymes. Stained with methylene blue. DNA for suspect J is Phi-X174 DNA. DNA for suspect K is the plasmid pBR322. For both gels: Lane C is undigested DNA, Lane B is BstN I digest, Lane H is Hae III digest, and Lane BP is 100 bp DNA ladder as reference for fragment lengths.

record of the separation. The location of the wells is traced first to aid in positioning the transparency film in case it slips. This transparency, or the gel itself, is now compared with "known" patterns of DNA fingerprints in order to determine the "identity" of the unknown sample and draw a conclusion about which of the fictitious suspects was present at the crime scene.

Further Details of Concern to Teachers

DNA Unknowns

We use DNA size standards with distinctive banding patterns that are commercially available, including the plasmid pBR322 digested with the restriction enzyme BstN I (New England Biolabs #303-1), DNA of the bacteriophage Phi-X174 digested with the restriction enzyme Hae III (New England Biolabs #302-6), 100 bp (base pair) DNA ladder (Gibco-BRL #15628-09) and 1 Kb (kilobase) DNA ladder (Gibco-BRL #15615-016). Students get dark bands with methylene blue stain using 2 μg of DNA (Figure 1). Advanced students use the restriction enzymes Hae III and BstN 1 to make their own digests of Phi-X174 DNA or pBR322 during a previous day's lab, leaving samples to digest overnight at room temperature (Figure 2). Life science or general science students use food color unknowns rather than DNA. We add glycerol to food color unknowns to increase their density (for loading) and add India ink to disguise their original colors. Food color unknowns do not require the addition of tracking dye, need only eight minutes for electrophoresis, and give easily compared two-color patterns that are visible immediately without staining and destaining (Figure 3). The food color patterns diffuse in a few hours. Therefore, they must be traced onto transparency film before the end of the period. (Student lab protocols and preparator's information are available from the authors upon request.)

The Combs

We built custom combs that stand on a tabletop (Bowlus & Grether 1994), but commercially supplied combs (such as Fotodyne #1-1417) perform satisfactorily if held by binder clips about 0.6 mm above the surface of the slide. (Three thicknesses of 3 × 5 card stock approximate 0.6 mm.)

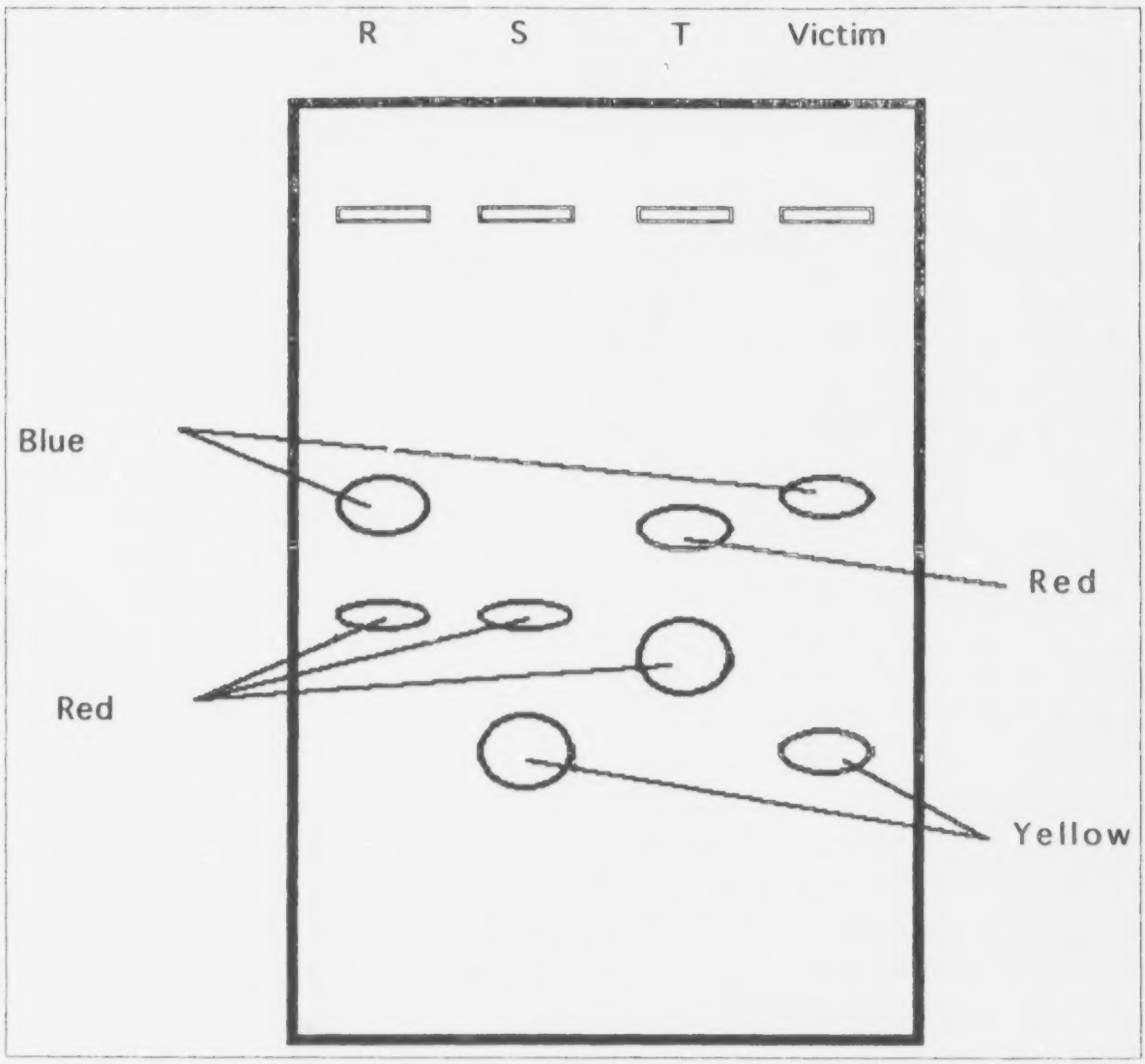

Figure 3. Electrophoresis patterns obtained with Schilling®-brand food coloring. No staining. Lanes as follows: Suspect R-Blue food color. Suspect S-Yellow food color. Suspect T-Red food color. Victim-Green food color.

Fitting the Gel to the Gel Box

If the gel is narrower than the width of the platform, a nonconducting block is set in place alongside the gel to displace buffer from the extra width, thus speeding electrophoresis through the gel for a given electric current (Figure 4). For our Fotodyne #El-1408 boxes (used without the gel tray) a 34 × 85 × 10-mm glass spot plate (VWR #53631-000) works as a space filler.

Loading the Gel

It is easier to see the wells for loading if the gel box rests on a dark-colored surface or if a black stripe about 2-cm wide has been painted on the underside of the gel platform.

Other Gel Configurations for Electrophoresis

Longer or wider gel boxes might accommodate more gels, but the time required for as many as eight students to load their wells would probably make the lab exceed the length of one class period.

Current During Electrophoresis

Currents higher than 100 milliamperes per gel, typically caused by

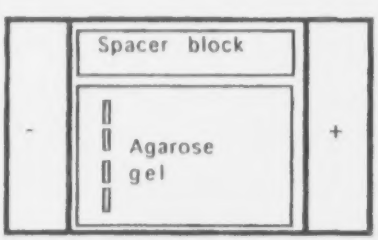

Figure 4. Top view of a wide gel box with a block of plastic or glass placed next to the agarose gel on the platform between buffer reservoirs. The gel is supported by a 50 × 75 mm glass slide.

excessive buffer depth over the gel, may lead to an overheated and possibly melted gel.

"Known DNA" Patterns

We suggest the students be given enlarged copies of the DNA fingerprints used in this lab exercise (Figure 1, 2 or 3, as appropriate). Because their gels cannot simply be superimposed, the students find it necessary to more thoughtfully observe and compare patterns in order to identify their unknowns.

Deionized Water

It may be possible to substitute tap water for distilled water. Test your local water for its effects on trial gels. The tap water at one of our schools renders the gels opaque and the bands invisible.

Applicability of Rapid Electrophoresis to Lambda-Virus DNA Samples

We obtained satisfactory results when we tested our rapid method with Lambda-DNA restriction fragments and 0.8% agarose.

Acknowledgment

Our work to develop DNA electrophoresis labs suited for high school use has been supported by W.M. Keck Foundation and National Science Foundation grants to the Center for Molecular Biotechnology at the California Institute of Technology.

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The Amylase Project

Creating a Classroom of Biotechnologists

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While working on the Amylase Project, your students' job is to produce the enzyme amylase in large quantities using microorganisms. (Amylase is an enzyme that breaks starch into sugar.) Your classroom becomes a biotechnology company and the students become scientists working towards a common goal. Their imaginary company may, for example, have a contract with Pepsi Cola™ to produce this enzyme so that high fructose corn syrup can be made efficiently from cornstarch. (High school students are familiar with soft drinks that use high fructose corn syrup as a sweetener). Other applications of amylase include enhancing the food removing ability of dishwashing and laundry detergents, increasing the smooth texture of wheat products, the desizing of fabric, and the removal of wallpaper.

The Amylase Project consists of four lab modules that can be done independently or in sequence. The modules incorporate concepts of microbiology, cellular biology, molecular biology, biochemistry, and evolution. They are all inexpensive to run (only one of the modules requires any special equipment; a UV light). Teachers can decide which of the "mini-labs" or modules to do. If all are completed, it is possible that some students could come up with a strain of bacterium which might be useful to a real biotechnology company. The following is an overview of the Amylase Project. A detailed instruction manual and plasmid DNA are available by writing the address given at the end of this article.

My past experience as a research associate in molecular biology at Genencor International (a biotechnology corporation in South San Francisco, CA) gave me practical knowledge along with an awareness of application which stretches beyond the classroom. While still at Genencor I became concerned about how little cutting-edge biology was being taught in high schools. I started a small program at the company to educate teachers about biotechnology. While working with this program, I realized that teaching was more suited to my personality than research. I have been teaching high school biology for six years now. During the summers, I began working on biotechnology experiments for high schools. My experience as a high school biology teacher has helped me to understand the limitations of a classroom lab, science department budgets, and the developmental stage of teenage minds. It is the combination of my experience in industrial biotechnology and in the high school classroom that makes these investigations unique. In November of 1992, I received the NABT/FOTODYNE Molecular Biology Teacher of the Year Award for the development of these investigative projects for students.

My goal for the Amylase Project was to try to distill the biotechnology process into a few short steps. Today, most biotechnology companies are involved in the production of proteins by microorganisms. The organism's DNA is usually altered (by mutation or transformation) and the organism is grown in such a way as to produce the most protein product possible. In the school setting, enzymes would be the easiest protein product to make and detect. Of the enzymes commonly produced by microorganisms, amylase is the easiest to detect, most easily understood by students, and has many applications in industry.

Module 1: Isolating Microorganisms That Naturally Produce Amylase

I usually start this module by telling the students that they are now scientists working for a biotechnology company. They have a contract with the soft drink industry to produce amylase. I ask them to tell me how they would go about producing amylase in large quantities. Invariably, I have a student who suggests that we hire hundreds of people to spit in a vat. Sometimes more practical students suggest extracting it from seeds. With some leading questions, I can usually guide them into thinking of bacteria or decomposers as being good producers of enzymes. I try to use this Socratic style throughout this project so that my students really do feel like scientists and not just recipe followers.

Summary

The "scientists" will isolate microorganisms from areas that they decide would have amylase-producing microorganisms. Likely choices might be on a potato, a rotting peach, or in the soil beneath a fruit tree. (Do not get samples from animals or humans.) They can streak these organisms on nutrient agar + 2% starch and see which organisms they isolated produce the most amylase. If an organism produces amylase, the starch in the surrounding agar will be broken down and a clearing zone around the colony will be present (Figure 1). If the organisms are growing too close together to see each distinctly, sterilized toothpicks can be used to spot a new plate so that the amounts can be more easily distinguished. A "bonus" of some sort could be given to the student with the organism that produces the most amylase.

The plates can be stained with iodine stain after the microorganisms have grown to see the starch breakdown more dramatically. The iodine will stain the starch in the agar purple or blue, while the colonies and the halos of starch degradation will remain unstained. The stained plates can be put on an overhead projector to show the whole class the differences between the sizes of halos (Figure 1).

Enrichment

In the process of this module, students can learn to see the difference between bacteria and fungi. (Molds are furry and filamentous with irregular edges while bacteria will have round, shiny colonies of white or yellow. Yeast also grow in shiny, circular colonies, but they can be bright orange.) It should become more clear how microorganisms are involved in the

Figure 1. Student results from Module 1: Sample from house plant soil.

decay process by the use of extracellular enzymes when the students see the starch being broken down by the amylase.

Module 2: Media Development—The Study of Repression & Induction

Summary

The next step for a scientist might be to change the media so that the organism will produce the most amylase possible. Usually, organisms will produce the most enzyme if the substrate of that enzyme is their only medium. In other words, a lot of glucose inhibits amylase production, while a starch medium (in the absence of glucose) tends to induce amylase production. The students can transfer their best organism onto agar with different constituents to see which medium is the best for the organism to grow quickly and make the most amylase. The teacher can make up a base agar that contains only the nutrients which the organism needs to survive + starch. This is called minimal starch media.

The students will add extra nutrients such as amino acids, vitamins, glucose, sucrose, minerals, salts, buffers at different pH's, etc. The students can think about what things the bacteria might need to grow healthily (and produce lots of protein) that isn't in the minimal medium. Have some solutions ready that the students can choose from, but allow them to be creative as well. One of my students, for example, brought in diet shake powder. These additives can be made into a solution and two drops can be spread onto the top of the minimal starch plate. Since the agar is minimal it does not support much growth, so little to no contamination will result. Also, since students are transferring their organisms from grown colonies (with a sterile toothpick), the growth of the chosen organisms will be far ahead of any growth that may occur from the unsterile nutrient solutions. Growth of a few contaminants may show up several days later, however. The student with the best improvement in halo size (and who can explain why his or her medium was the best) gets a bonus (Figure 2).

Enrichment

If the students have learned or are going to learn about the tryptophan (trp) operon, or other operon systems in bacteria, this is an excellent lab to show how repression works in bacteria. Agai with lots of glucose will inhibit amylase production. Students can hypothesize what might be happening on the molecular level. One hypothesis could be that glucose binds to and activates a repressor of the amylase gene.

Conceptual Cautions

Students may become confused and develop a Lamarckian view of what is happening. A clear discussion of gene regulation is needed. In this module, the DNA is not changing, but different genes are being expressed to different degrees. Also, be sure to distinguish between growth rates of the bacteria and amylase production. Glucose will speed the growth of organisms, but may repress amylase production. So you may see a larger colony sooner, but proportionately the amount of amylase produced per cell may be less.

Module 3: Ultraviolet Mutagenesis

Summary

This experiment will require either a hand-held UV light or a UV light transilluminator such as those used for agarose gel electrophoresis. FOTODYNE makes a UV light box (short wave: 320 nm) for gel electrophoresis which works very well. Sargent-Welch makes a less expensive UV light that works well also.

The FOTODYNE UV light box is equipped with a Plexiglas™ shield. The light will not turn on unless the shield is covering the UV light source. If you are not using this equipment, special UV protecting glasses should be worn. I have calibrated the experiment for the FOTODYNE and the Sargent-Welch machines. You will need to experiment with other UV sources to determine the best amount of time to mutate.

The experiment involves mutating the *Bacillus subtilis* with ultraviolet light. I suggest using this type of bacterium because it is well studied, and both up and down mutations have been observed in this strain. Also, the type of medium, growth and mutation times for *Bacillus subtilis* have been measured and are given in the *Amylase Project Manual*. It is very possible that the amylase-producing organisms the students isolated are in fact *Bacillus subtilis*, since *Bacillus subtilis* is very common in soil. As a transition from module one or two, you can tell the students that the best producer was chosen, and mutagenesis will be done

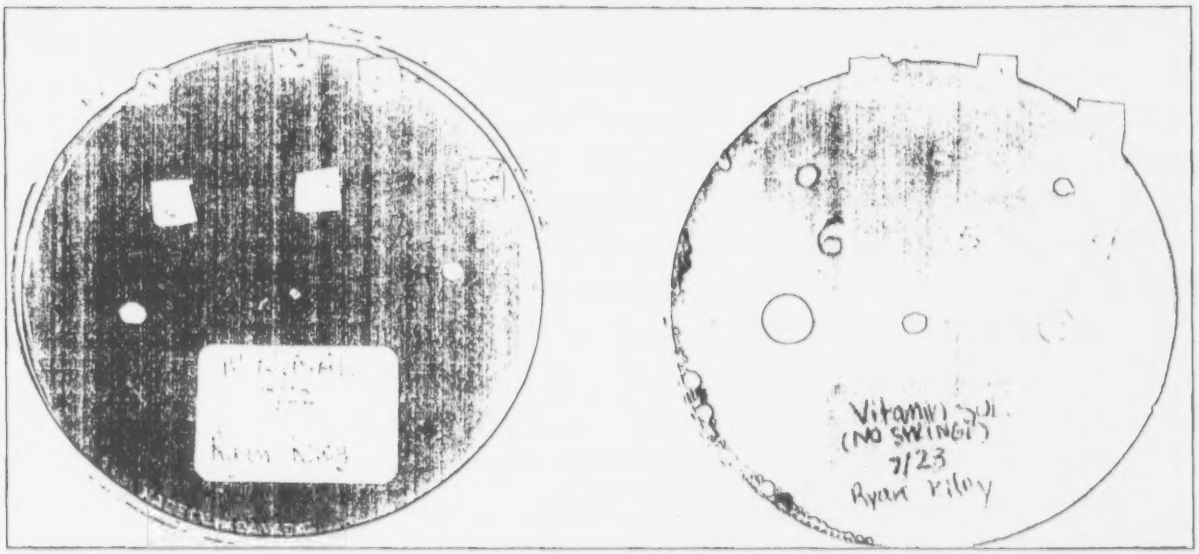

Figure 2. Student results from Module 2: Notice the difference the addition of vitamins makes to amylase production.

on this strain only. (This is often the case in industry.) The bacteria are grown up in liquid culture and plated on nutrient agar + starch. The plates are exposed to the UV light. Most (99.99%) of the bacteria will be killed by the UV light. The survivors (10–100 colonies) will be mutated in several locations on their chromosome. The mutants generally produce varying amounts of amylase. Most of the mutations cause less amaylase to be produced (down mutation), but once in a while (at least one per class), a mutation will cause the bacterium to produce more amylase, an up mutation (Figure 3).

Enrichment

This shows the effects of UV light on DNA as well as reinforcing the concept that a change in DNA will result in a change in the activity of an organism. This is also a great demonstration of mutation and natural selection. The mutation and reproduction rates are artificially increased, and the organisms are selected on the basis of amylase production. Also, a discussion of skin cancer and its causes can be raised. When the *Bacillus* has grown in liquid culture before mutagenesis, students can look at a drop of the culture under a microscope. *Bacillus* is rod shaped and motile. If the culture is in log phase growth, long chains will be seen. If it is in stationary phase, single motile cells will be seen. After a week or so, spores will be seen. *Bacillus* cells, especially in log phase growth, are especially easy to see under a microscope (400× is plenty) because the chains are so long.

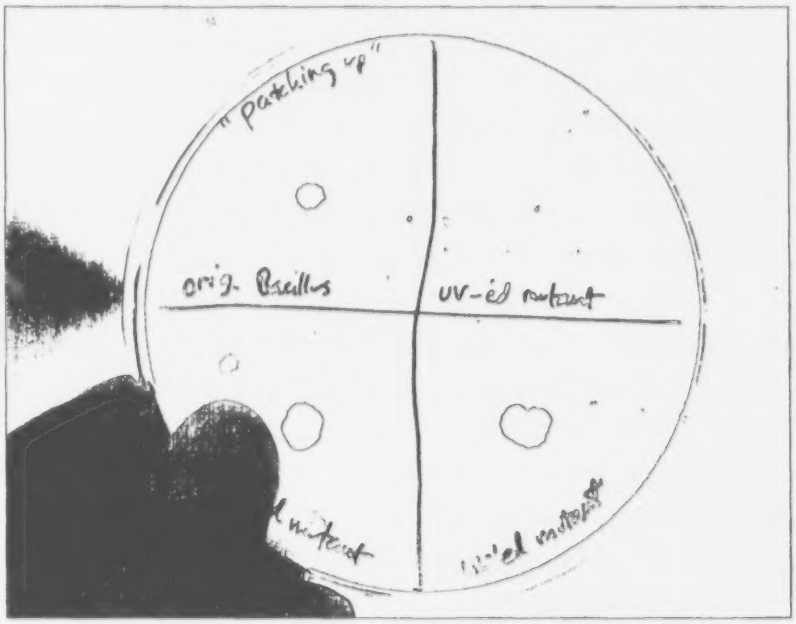

Figure 3. Student results from Module 3: Mutants of *Bacillus* patched to a new plate to observe the differences better. The unmutated *Bacillus* before mutation is in upper left quadrant.

Module 4: Transformation of E. Coli *with the Amylase Gene from* Bacillus

Summary

If a native organism is not making enough of the enzyme you want, what do you do? Move the gene to another organism! *E. coli* grows vigorously and it is often the organism of choice. In this module, students will move a *Bacillus* amylase gene into *E. coli*. In this way, they will produce a true recombinant organism. Having students clone the amylase gene from *Bacillus* would be too time consuming, so the students will only do the last, but most exciting step.

This module simply involves the transformation of *E. coli* with an amylase gene that is carried by a plasmid with antibiotic resistance, pUC18. (Genencor International cloned the amylase gene from the chromosome of *Bacillus*. This gene was then inserted into the plasmid pUC18.) Plasmids are small circular pieces of DNA that occur naturally in bacteria. Plasmids are convenient shuttles for transporting genes into bacteria. The gene of choice is inserted into a plasmid that also contains a gene for resistance to an antibiotic; then the plasmid is taken up by bacteria. Selection for only the bacteria that accepted the plasmid is done by spreading the bacteria on agar which contains antibiotics. Because the antibiotic resistance gene on the plasmid will enable the bacteria to survive on antibiotics, only the bacteria that received the plasmid will survive.

In our case, all of these survivors will now contain the amylase gene as well. These newly "transformed" bacteria will now produce the protein product of that gene, which is the enzyme amylase. The *E. coli* should be treated with $CaC1_2$ before the plasmid is added. I have designed a quick and easy way to make highly competent cells in the high school classroom. With this method, 100% of your students should get results. The *E. coli* will remain "competent" (able to be transformed) for up to one week if they are kept in a refrigerator (4° C). The transformation of *E. coli* with this particular plasmid is perhaps the most dynamic part of this amaylase project. Most other transformations that are done in school settings only use "empty" (no gene insert) plasmids. This plasmid contains an amylase gene so that students will be producing a marketable product. The fact that the cells couldn't make amylase before and couldn't grow in the presence of antibiotics and now they can produce sizable amounts of amylase and survive on agar containing antibiotics makes the effect of adding the genes obvious and unforgettable (Figure 4).

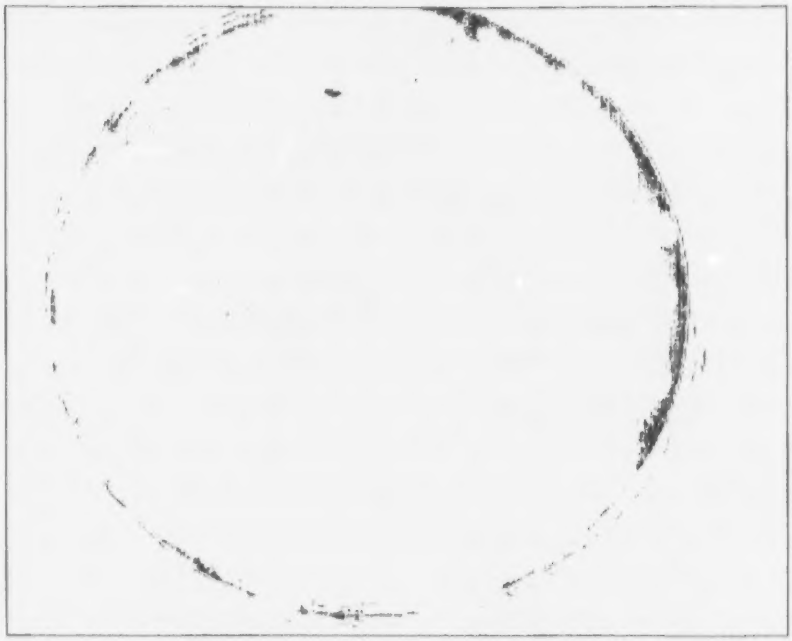

Figure 4. Student results from Module 4: Amylase producing *E. coli* transformants.

The Amylase Project Manual and amylase plasmid DNA are available through Flinn Scientific, (800) 452-1261.

DNA Fingerprinting in a High School Research-Based Science Course

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The DNA profile technique, more commonly referred to as DNA fingerprinting, has vaulted into the public eye as a result of the intense media coverage of numerous criminal cases. This relatively new and still somewhat controversial technique is used to link a suspect to biological evidence from a crime scene, or to resolve paternity disputes. DNA profile analysis provides an exciting opportunity for students to learn more about the genetic variability among members of a population and to explore important laboratory techniques such as the Polymerase Chain Reaction (PCR) and gel electrophoresis.

The DNA profile technique was performed by a group of high school juniors and seniors. All of the students had completed biology and chemistry courses, but had no other specialized training. The class consisted of 10 males and 12 females. Seventy percent of the students successfully determined their own DNA profiles using the procedures presented in this article.

A Brief Guide to DNA Profile Analysis

VNTR Loci

Each human somatic cell nucleus contains 6.4 billion base pairs of DNA. Portions of this DNA encode over 100,000 genes, while the remainder of the DNA is non-coding. Among all individuals, 99.9% of human DNA is identical. The remaining 0.1% can differ in several ways between individuals. DNA profile analysis focuses on hyper variable regions of DNA. One such form of variability is called VNTR (variable number of tandem repeats).

One DNA segment (locus), a VNTR denoted D1S80 (Kasai et al. 1991), was analyzed in this study. D1S80 is located on human Chromosome 1 (Figure 1). This locus is composed of repeating units of segments of DNA that are 16 nucleotides—long (Figure 2). The number of repeats varies from one individual to the next, and is known to range from 15 to over 41 (Budowle et al. 1995). This variability in the number of repeats is the basis for DNA profile analysis.

Methods

Determining the number of repeats requires three general steps:

1. Obtain a sample of human DNA.
2. Make many copies of specific VNTR DNA from the sample.
3. Observe the amplified VNTR DNA.

Obtaining the DNA Sample

Human DNA can be obtained from a variety of sources including saliva, blood, hair, skin and seminal fluid. The high school students isolated DNA from saliva using a modified version of the procedure described by Walsh et al. (1991).

1. Saturate a clean cotton swab with saliva by gently wiping the inside of the cheek.
2. Cut off the tip of the saturated swab and place it in a clean 1.7-ml microcentrifuge tube.
3. Add 500 microliters of 5% (w/v) Chelex® to the sample (5 g Chelex® to 100 ml H_2O).
4. Incubate at 56° C for 30 minutes. The swab tip may be removed after incubation and placed in a 10% bleach solution prior to disposal.
5. Vortex the sample for 10 seconds.
6. Incubate in a boiling water bath for 8 minutes.
7. Vortex the sample for 10 seconds.
8. Spin in a microcentrifuge at maximum speed for 3 minutes.

There should be two distinct layers visible in the reaction tube at the completion of Step 8. The bottom layer contains the 5% Chelex and extraneous cellular matter, while the top layer contains an aqueous solution of DNA. The sample is now ready for PCR amplification, or may be stored temporarily at 4° C or frozen at −20° C. To reuse these samples after storage, thaw if necessary and repeat Steps 7 and 8.

PCR Amplification of the D1S80 VNTR Locus

A detailed discussion of PCR is described by Garrison and dePamphilis (1994). The following procedure, which yields approximately 10^9 copies of the D1S80 locus, was performed by the students.

1. For each PCR reaction, the following components are added to a 0.65-milliliter microcentrifuge tube.

4 µl	50 mM $MgCl_2$
2 µl	10 mM dNTP Mix
2 µl	0.2 mg/ml Primer 1
2 µl	0.2 mg/ml Primer 2
60 µl	H_2O
20 µl	DNA
90 µl	Subtotal Volume

*Primer sequences are underlined and indicated in bold type in Figure 2.

2. Place one AmpliWax™ (Perkin Elmer) pellet into each reaction tube. Heat the tube to 65° C for 1 minute, then allow to cool to room temperature. The wax forms a barrier on top of the 90 µl reaction mixture.
3. Dilute Taq polymerase enzyme with 10x PCR buffer to a final concentration of 2.5 units/10 µl PCR buffer.
4. Add 10 µl of the diluted Taq polymerase to the top of the wax barrier. (Do not pierce the wax!)
5. Place the reaction tubes into a thermal cycling machine programmed

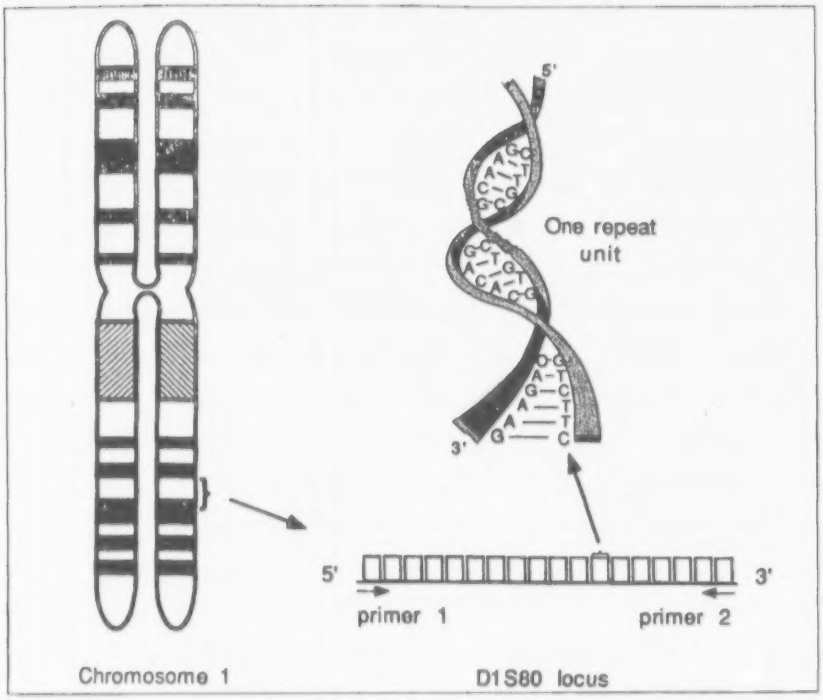

Figure 1. A schematic representation of Chromosome 1 and the D1S80 locus. Boxes represent tandem repeat units, each being 16 nucleotides long. One repeat unit is shown as a double helical structure.

to run 29 cycles with the following parameters:

Denaturation	94° C for 1 minute
Annealing	65° C for 1 minute
Extension	72° C for 1 minute

6. Completed reactions may be stored at −20° C indefinitely.

Observing DNA Using Agarose Electrophoresis

Once the PCR reaction is completed, the DNA is ready for analysis using gel electrophoresis. For each sample to be analyzed, penetrate the wax barrier with a sterile micropipet tip; remove 20 microliters of the PCR product and place it in a fresh microcentrifuge tube. Combine each sample with 2 µl of loading buffer (30% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue in H_2O) and place into adjacent wells on a 2% agarose gel in 1 × TAE (40 mM Tris acetate, 1 mM EDTA). A size marker should be loaded into a well adjacent to the samples. (We used the bacteriophage ϕx174 digested by the enzyme Hae III.) Electrophorese the DNA at 60 volts until the bromophenol blue indicator in the loading buffer is 2 cm from the bottom of the gel (approximately 1.5 hours for a 10-cm long, 0.4-cm thick gel).

Remove the gel from the electrophoresis apparatus and place it in a container of dilute ethidium bromide 1 µg/ml). After 10 minutes, the gel is ready for viewing under ultraviolet light. The gel may be photographed for later analysis.

Allele sizes can be estimated by comparing them to the size marker. A more accurate determination of allele size can be made using the AmpliFLP™ allelic ladder provided in Perkin Elmer's AmpliFLP™ D1S80 kit, or may be ordered separately from Perkin Elmer.

Obtaining Reagents & Equipment

Equipment required for DNA profile analysis that might not be found in the typical high school laboratory is available through scientific supply houses such as VWR, Fisher Scientific, and Carolina Biological. We purchased enzymes and other reagents from Sigma Chemical Co., Life Technologies Inc. (formerly Gibco BRL), Bio-Rad, and Perkin Elmer. The following is a list of equipment and supplies used in this experiment. Sources for some items are listed in parentheses.

- Micropipets: 20 µl, 200 µl and 1000 µl capacities
- Micropipet tips
- Microcentrifuge tubes, 0.65 ml and 1.7 ml capacities.
- Microcentrifuge
- Cotton swabs
- Chelex® 100 resin, biotechnology grade (Bio-Rad)
- Vortex test-tube mixer
- Taq polymerase, patented by Hoffman-LaRoche. Comes with 10× PCR buffer and 50 mM $MgCl_2$. (Life Technologies, Inc.)
- 10 mM dNTP mix (Life Technologies, Inc.)
- PCR primers 1 and 2 for D1S80 locus
- AmpliWax™ wax pellets (Perkin Elmer)
- Electrophoresis power supply
- Submarine gel kit, including tank tray and combs
- Ultraviolet illuminator
- Agarose
- 1 × TAE: Tris acetate and EDTA
- Ethidium bromide
- Gel loading buffer: glycerol, xylene cyanol and bromophenol blue
- DNA size marker: ϕx174-Hae III or AmpliFLP™ allelic ladder
- Thermal cycler

Perkin Elmer sells a PCR amplification kit under the name AmpliFLP™ D1S80 (part no. N808-0054) which contains all reagents, tubes, marker and control DNA required to perform 50 PCR amplifications of the D1S80 locus for $425.

The thermal cycling machine used by the Sahuaro High School students was from the senior investigator's laboratory at The University of Arizona. This machine, a Gene-E thermal cycler, is available from VWR Scientific for $1600 with an institutional discount. Manual PCR reactions without the aid of a thermal cycling machine are discussed by Garrison and dePamphilis (1994) but were not attempted in this study.

Classroom Implementation

Students must be advised of the risks associated with fluid-borne pathogens and instructed in proper hygiene so as to avoid infection. They are required to wear latex gloves throughout the experiment. Biohazard waste (e.g. used swabs and microcentrifuge tubes) must be placed in a 10% bleach solution prior to disposal. Ethidium bromide is a neurotoxin and potential carcinogen. Avoid contact with the skin and dispose of as a hazardous organic compound. Flinn

Figure 2. DNA sequence of D1S80 (Kasai et al. 1991). A human D1S80 locus containing 18 tandem repeats is shown. The most common alleles are either 18 or 24 repeats (Sagantila et al. 1992). Oligonucleotide primer sequences are underlined and indicated in bold type.

Scientific, Inc. (P.O. Box 219, Batavia, IL 60510) publishes a catalog which contains information on disposal of hazardous substances.

Correct use of micropipetors is perhaps the most problematic task encountered in the experiment. Careful instruction in the proper use of the micropipetors is essential. To avoid waste of expensive reagents, a pipeting practice session using water is advisable. Additional practice manipulating DNA is encouraged. A useful practice experiment might include cutting plasmid DNA (we used pBlue-script KS+) with a restriction endonuclease. The resulting DNA segments of known size can be analyzed by agarose gel electrophoresis.

Following the practice activities, students are prepared to begin the DNA fingerprinting activity. Results obtained by the Sahuaro High School students are shown in Figure 3.

Student Projects

In one project, students are provided with a sample of DNA obtained from a mock crime scene. Using DNA profile analysis, they can then determine who among them might be the perpetrator of the crime. In another project, students can determine D1S80 allele frequencies among schoolmates. A database can then be constructed and comparisons made to known allelic frequencies.

Acknowledgments

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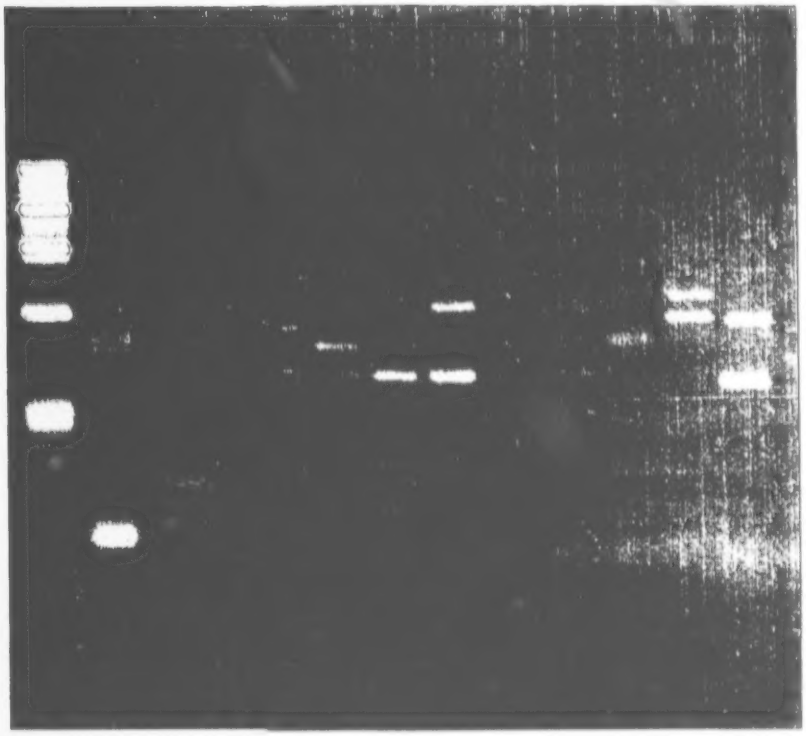

Figure 3. Student DNA fingerprint data. Lane 1 contains DNA size markers (ϕx174-Hae III). Lanes 2–15 contain 14 different student samples. Bands represent alleles received from each parent. Individuals demonstrating a single band are homozygous, having received the same number of tandem repeats from both parents.

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An Authentic RFLP Lab for High School Or College Biology Students

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Restriction fragment length polymorphism (RFLP) analysis was first described in the mid-1980s (Jeffreys et al. 1985a, b). This technique (sometimes called DNA fingerprinting) allows for the identification of DNA sequence differences in individuals, based on the presence or absence of restriction enzyme sites in the genome of those individuals. Polymerase Chain Reaction (PCR) methods are becoming more widely used for DNA fingerprinting (Kloosterman et al. 1993), but RFLP analysis is still widely used in forensics, paternity cases, ecological studies, mutation identification, and many other areas of biology (Old & Primrose 1994).

RFLP analysis has also become widely used in high school and college teaching laboratories. However, the technique as practiced by many of these students does not accurately portray RFLP analysis. Actual RFLP analysis relies on digesting different genomes with the same restriction enzymes. RFLP exercises in teaching laboratories are frequently simulated by digesting the same genome with two different restriction enzymes (Hepfer et al. 1993) or by digesting different genomes with different restriction enzymes (Bowlus & Grether 1996). While these procedures produce different sized bands on an agarose gel, they do not accurately portray the underlying basis of the technique, which relies on comparing different genomes digested with the same restriction enzyme.

There is at least one authentic DNA fingerprinting analysis laboratory exercise that is readily available for classroom use (Modern Biology Inc. 1996). This kit uses sheep and cow genomic DNA and allows students to observe differences in highly repeated DNA sequences in the two species. The exercise we describe here is an alternative, authentic DNA fingerprinting analysis.

This RFLP exercise involves digestion of different bacteriophage λ genomes with the same restriction enzyme. Bacteriophage λ and bacteriophage λgt10, which infect *E. coli*, are used in this exercise. λgt10 is derived from wild-type λ and is specifically constructed so that it contains a single *Eco*RI site (Huynh et al. 1985). In contrast, wild-type λ phage contains five *Eco*RI sites (Sambrook et al. 1989) (Figure 1). The difference in sizes of *Eco*RI fragments from the two different bacteriophage genomes can be readily observed after agarose gel electrophoresis (Figures 2, 3). The exercise can be done either as a means of simply showing RFLPs, as a simulated molecular epidemiology laboratory, or as a simulated forensic laboratory exercise.

With the appropriate guidance from the instructor, this exercise can also help high school students understand the content underlying several national standards. For example, one of the standards requires understanding that, "The information passed from parents to offspring is coded in DNA molecules" (AAAS 1993, p. 108). In this exercise, students are digesting thousands of DNA molecules, each of which has an identical (or nearly identical) sequence to the parental phage from which it was derived. Another one of the standards requires understanding that, "Genes are segments of DNA molecules. Inserting, deleting, or substituting DNA segments can alter genes" (AAAS 1993, p. 109). In this exercise, students are directly examining alterations in DNA that lead to an observable difference; in this case, a different restriction enzyme digestion profile.

Materials

- **DNA.** λgt10 and wild-type bacteriophage λ were purchased from Promega Biotech, Madison, WI. These DNAs are also available from Sigma Chemical Co., St. Louis, MO and other suppliers.
- **Restriction Enzyme.** *Eco*RI was purchased from New England Biolabs, Beverly, MA, Promega Biotech, or Sigma Chemical Co.
- **Agarose Gels and Buffers.** Agarose was obtained from FMC Bioproducts, Rockland, ME, or Fisher Scientific, Chicago, IL. 0.5× TBE (0.045 M Tris-borate, 0.001 M EDTA, pH8) was used as the electrophoresis buffer. It was diluted with distilled water from a 5× stock of TBE buffer. The 5× TBE buffer was prepared by adding 54 g of Tris base, 27.5 g of boric acid, 20 ml of a 0.5 M EDTA solution (pH 8), and deionized water to a final volume of 1 liter (Sambrook et al. 1989). Pre-mixed TBE solutions are also available commercially. 30 ml, 0.6% gels were cast and run in a B1A Owl Easy-cast mini-gel system with a 7 cm × 8 cm gel and a comb with 6 teeth (9 mm × 1.5 mm) or 10 teeth (5 mm × 1.5 mm) (Owl Scientific Products, Woburn, MA).
- **Nile Blue Staining.** Nile Blue stain (Sigma, N-3632) was dissolved in distilled water at a concentration of 2 mg/ml (100× stock). It is likely that the diluted staining solution can be reused several times, but we have not tested this.
- **Ethidium Bromide Staining.** Ethidium bromide stock solution was prepared in water at a concentration of 5 mg/ml. Three µl of this solution was added to a 30 ml agarose gel prior to electrophoresis.

Lab Outline for RFLP Analysis Using Nile Blue Staining

Pre-Lab DNA Preparation

Dilute λ and λgt10 DNA in separate tubes with TE (10 mM Tris, pH8, 0.1 mM EDTA, pH8) so that 17 µl of the solution contains 0.5 µg of DNA (for example, if the stock of λ DNA contains 500 µg of DNA per ml, then 1 µl of that solution will contain 0.5 µg, so the diluted solution would be prepared by mixing 1 µl of λ DNA with each 16 µl of TE). Make a large

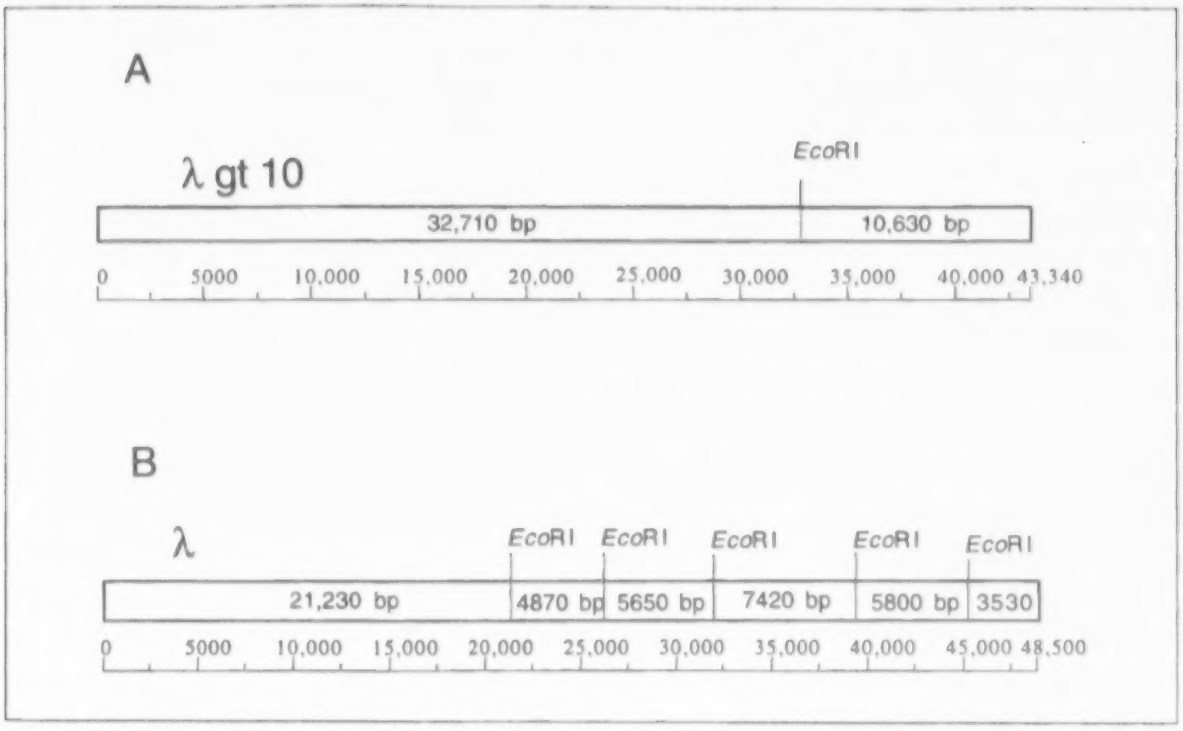

Figure 1. Restriction enzyme maps of λgt10 and λ. The boxes represent the bacteriophage genomes. The numbers in the boxes indicate the sizes of the restriction enzyme fragments generated by *Eco*RI restriction enzyme digestion. The numbers on the graph below the boxes indicate the numbers of base pairs from the lefthand side of the phage genomes. *Eco*RI indicates the location of *Eco*RI restriction enzyme sites. A) λgt10 map; B) wild-type bacteriophage λ map. The maps are based on information from Sambrook et al. (1989).

enough batch of the diluted DNA so that each student or group of students will have 17 µl of λ and λgt10 DNA solution. The diluted DNA can be stored for at least a year in a −20° C freezer. Aliquot sufficient restriction enzyme buffer and restriction enzyme for the class into several microcentrifuge tubes. This helps to ensure that the stock solutions are not contaminated due to students accidentally pipeting another solution into the enzyme or buffer stock solutions.

Day 1

Restriction enzyme digest. Each group of two students is given two 0.5-ml or 1.7-ml microcentrifuge tubes that will be used for the restriction enzyme digestion. One tube should be marked "λ," the other "gt10." Students add 2 µl *Eco*RI restriction enzyme buffer and 1 µl *Eco*RI restriction enzyme (7–15 units) to each tube. Then they add 17 µl of DNA solution (prepared as described above) containing λ DNA to the appropriate tube and 17 µl of λgt10 DNA to the other tube, for a final volume of 20 µl in each tube. This solution is incubated for one hour to overnight at 37° C in an air incubator. If a microcentrifuge is available, briefly centrifuge the tubes after digestion, particularly if any condensation is visible in the cap of the tubes. (Samples can be frozen at −20° C for at least a few weeks after digestion, if necessary.)

Before running and staining the gel, students are given the restriction enzyme sites for each genome (Figure 1) and are asked to determine the fragment lengths and sketch their expected gel results.

Day 2

Pour, run and stain agarose gels. For a 7 × 8-cm gel, we used 30 ml of 0.6% agarose, with a 10-tooth comb (5 mm × 1.5 mm) per well. (Larger combs can be used, but proportionately more DNA should be used to ensure that the DNA bands can be seen on the gel.) The gel is made by adding 0.18 g of agarose to 30 ml 0.5× TBE to a loosely capped bottle. The mixture is microwaved at 50% power for approximately 50 seconds, with the bottle swirled every 5–10 seconds to prevent boilover. The gel is poured with a comb in place and allowed to solidify (about 45 minutes to one hour at room temperature). It is very important to make sure the gel solidifies completely before removing the comb. If the comb is removed too early, the wells may deform, and the DNA may run as distorted bands on the gel. To ensure integrity of the wells, we also find it helpful to pour buffer over the solidified gel before removing the comb. If necessary, the gel can be poured the previous day and covered with electrophoresis buffer once it solidifies, or wrapped in plastic wrap and refrigerated (this helps prevent the gel from drying out). "Mini-gel" methods would probably also work well for this exercise (e.g. Bowlus & Grether 1996). When the gels are poured with a 10-well comb, five pairs of students can run their samples on a single gel.

Once the agarose has solidified, the gel is covered with 0.5× TBE buffer

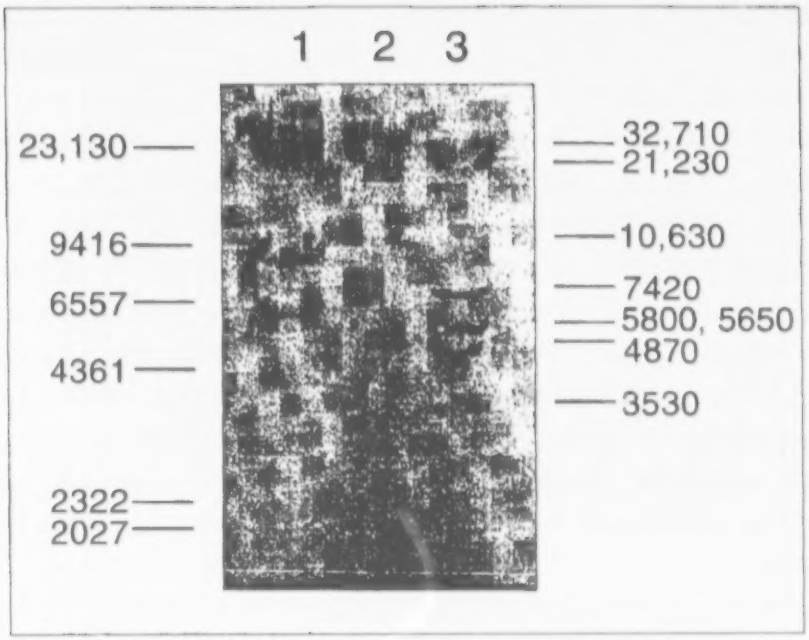

Figure 2. Nile blue stained agarose gel of *Eco*RI-digested λ and λgt10. Lane 1: 0.5 μg of λ digested with *Hind* III (size marker). Lane 2: 0.5 μg of λgt10 digested with *Eco*RI. Lane 3: 0.5 μg of λ digested with *Eco*RI. Numbers to the left of the gel indicate the sizes in base pairs (bp) for DNA fragments resulting from the *Hind* III digest of λ. Numbers to the right of the gel indicate bp sizes for DNA fragments resulting from the *Eco*RI digest of λ and λgt10.

and the comb is removed. If a water bath is available, the DNA samples should be heated at 60° C to 65° C for 3 minutes (see Notes section on p. 89) and cooled on ice for 2–3 minutes. Students then add 2 μl of loading dye to their samples [loading dye is available commercially from a number of suppliers or can be prepared as described by Sambrook et al. (1989) or FMC Bioproducts (1997)]. Then students can carefully pipet 20 μl of the DNA samples into the wells with a micropipeting device. Explain to the students that the tip of the pipetor should be placed just into the top of the well and that they should be careful not to put the pipetor into the well too deeply or they risk puncturing the well and losing their sample. The loaded gels are then electrophoresed at 50–60 volts for 1–3 hours. As an alternative, the gel can be electrophoresed overnight at 15 volts. The bromophenol blue dye (dark blue) should have migrated at least half the length of the gel before electrophoresis is terminated; longer runs, where the bromophenol blue dye migrates two-thirds to the full length of the gel, will typically show better separation of the DNA bands.

After electrophoresis, the gel is carefully placed in a plastic staining tray (e.g. Rubbermaid sandwich container) that contains 3 ml of the 2 mg/ml Nile Blue dye solution in 300 ml of deionized water, and the gel is stained for one hour to overnight at room temperature. The gel is then placed in deionized water for storage and destaining. This destaining step increases the visibility of the DNA (although the DNA bands are typically visible without destaining). The DNA is visible for at least four months when the gels are stored, after staining, in distilled water at 4° C. An example of the results from this method is shown in Figure 2. Note the clear difference in DNA fragment sizes between the wild-type bacteriophage λ and λgt10.

After the gel has been run and stained, students copy the bands present in their gel onto a transparency placed over the gel. They then compare the banding pattern they predicted prior to running the gel with the banding pattern actually generated. The key thing is for students to compare the relative position of bands between the two DNA samples (e.g. that the 10,630 bp band from λgt10 would be above the 7420 bp band from λ) since the actual position of bands will vary depending on how the gel was run. Note that with the gel system that we used, the 5800 and 5650 bp λ DNA bands are not separated. Therefore, the λ DNA shows five, rather than six, bands on these gels.

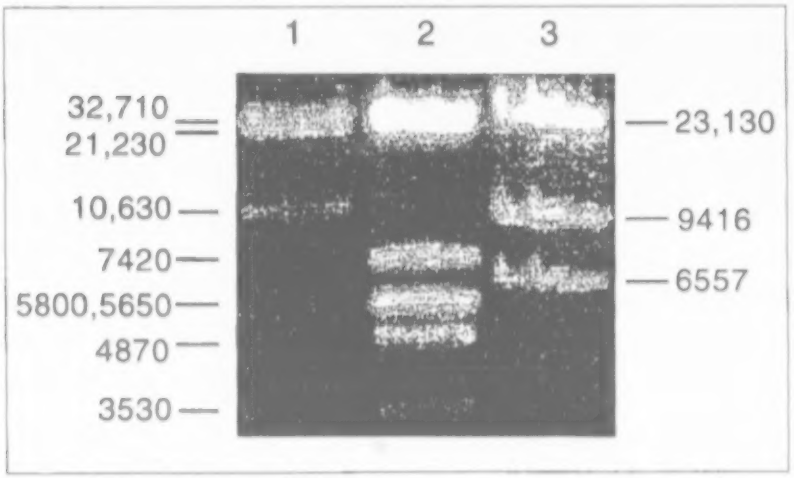

Figure 3. Ethidium bromide stained agarose gel of *Eco*RI-digested λ and λgt10. Lane 1: 0.1 μg λgt10 digested with *Eco*RI. Lane 2: 0.2 μg λ digested with *Eco*RI. Lane 3: 0.375 μg λ digested with *Hind* III (size marker). Numbers to the right of the gel indicate bp sizes for DNA fragments resulting from the *Hind* III digest of λ. Numbers to the left of the gel indicate bp sizes for DNA fragments resulting from the *Eco*RI digest of λ and λgt10.

Lab Outline for RFLP Analysis Using Ethidium Bromide Staining

Pre-Lab DNA Preparation

Dilute λgt10 DNA in TE (10 mM Tris, pH8, 0.1 mM EDTA, pH8) so that

17 µl of the solution contains 0.2 µg of DNA. Dilute λ so that 17 µl of the solution contains 0.2 µg of DNA. The resulting solutions can be stored for at least a year in a −20° C freezer.

The procedure is the same as that for Nile Blue staining, except that only 0.2 µg of λgt10 DNA and λ DNA is used, 3 µl of a 5 mg/ml ethidium bromide solution is added to the gel, and a 6-well comb is used (9-mm × 1.5-mm teeth). The DNA bands are visualized under UV light, and may be photographed with the appropriate camera (we use Polaroid Type 667 film in a Fotodyne FCR-10 camera [Fotodyne, Hartland, WI]. We have been able to detect the 10,630 bp DNA band in λgt10 DNA when as little as 0.025 µg of *Eco*RI-digested λgt10 was loaded on a gel, but we prefer to use 0.2 µg of λgt10 DNA to ensure that the bands are readily visible to students.

The ethidium bromide staining method has the advantage of being able to detect a smaller amount of DNA, which lowers the cost of the laboratory exercise considerably. The drawbacks are the handling and disposal problems associated with working with ethidium bromide, a toxic, mutagenic compound, and the expense and potential hazard of using a UV transilluminator. The results from an ethidium bromide-stained gel are shown in Figure 3. Again, note the clear size distinction between the lower molecular weight DNA bands from the λ and λgt10 digests.

Alternative Formats for the Laboratory Exercise

This exercise, using either staining method, could be turned into a molecular epidemiology study. λgt10 could represent one known virus strain; λ a second, more virulent strain of the virus, and the unknown (either λ or λgt10) could represent a virus isolated from a patient in the hospital. Students would do the digests, then run their knowns and unknowns on a gel. Based on which unknown they had, they could form a hypothesis about the likely virulence of the virus from the patient. An example of the results from this version of the laboratory exercise is shown in Figure 4.

A further variation would be to do this exercise as a forensic simulation by including known λ and λgt10 DNAs as above and then an "unknown" DNA from the crime scene. For example, the λ DNA could represent the suspect; the λgt10 the victim; and the unknown could be a sample of the λ DNA. After the digestion and electrophoresis, it should be clear to the students that the "suspect's" DNA matches the DNA found at the "crime scene."

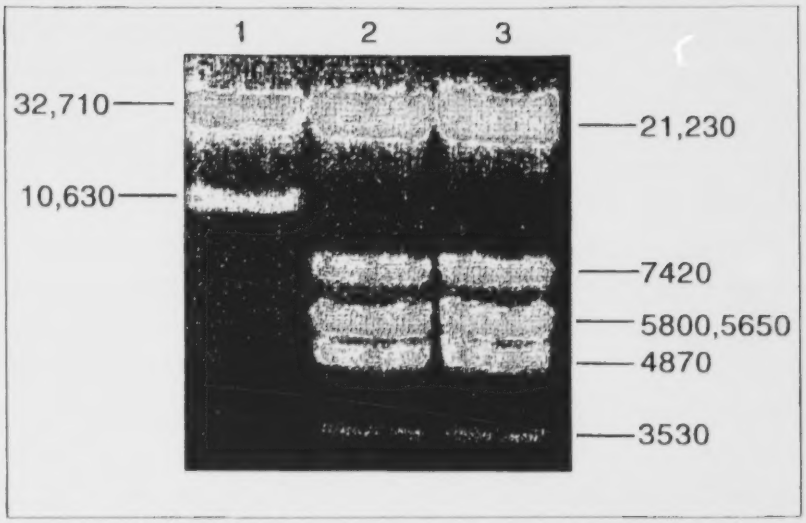

Figure 4. Simulated molecular epidemiology analysis. Lane 1: DNA from a less virulent strain of the virus (λgt10, 0.1 µg, *Eco*RI digested). Lane 2: DNA from a more virulent strain (λ, 0.2 µg, *Eco*RI digested). Lane 3: DNA from a virus isolated from a "patient" (λ, 0.2 µg, *Eco*RI digested). The numbers to the left of the gel indicate bp sizes of DNA fragments resulting from the λgt10 digest, the numbers to the right of the gel indicate bp sizes of DNA fragments resulting from the λ digest.

Results

This lab exercise was done by 65 students in three sections of a high school biotechnology course at Bemidji High School, Bemidji, MN. Ninety-one percent (59 students) were able to observe the RFLP differences between λ and λgt10 in their samples.

Notes

As an alternative, λgt11, which also has a single *Eco*RI site, could also be used in this exercise. λgt11 can be purchased from a number of vendors, including Promega Biotech and Sigma Chemical Company.

An air incubator is preferred over a water bath for long (i.e. more than one hour) incubations. Longer incubations in a water bath tend to cause more condensation on the top of the tubes, which changes the buffer composition in the bottom of the tube and could affect digestion by the restriction enzyme.

As noted in the description of the exercise for Day 2, we recommend heating the digested DNA prior to loading the gel. The heating step disrupts hydrogen bonds between the cohesive ends of lambda DNA (these cohesive ends are located at each end of the λ DNAs shown in the map in Figure 1). The exercise can be done without this step, but the λgt10 DNA band of 10,630 bp and the λ DNA band of 3530 bp will be faint, because those DNAs will bond to, and run with, the largest DNA fragments from those digests. A clear illustration of this is in Figure 2, Lane 1. The 4361 bp fragment from the *Hind* III digest of lambda contains a cohesive end and anneals to the cohesive end of the 23,130 bp fragment in that digest. Thus the 4361 bp band is lighter than the 2027 bp band, even though it should be twice as dark.

Methylene blue stain, as well as commercial products from Carolina Biological Supply (Burlington, NC), Wards (Rochester, NY), Bio-Rad (Hercules, CA), Modern Biology (West Lafayette, IN) and others are widely available for staining DNA in gels. We found that Nile Blue staining was more sensitive than methylene blue staining and was less expensive than commercially available stains which had approximately the same sensitivity. The use of Nile Blue for staining DNA was previously described by Adkins and Burgmeister (1996). Our procedure differs somewhat from their method in that we stain the DNA after, rather than during, gel electrophoresis.

TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) can be used in place of TBE buffer for electrophoresis. TAE

gives better separation of large DNA fragments, but it has limited buffering capacity, which may require that the buffer be recirculated for electrophoresis experiments that take longer than 6 hours (FMC Bioproducts, 1997).

The 0.6% agarose gels used in this exercise are quite fragile. These gels should only be transferred with a spatula or other device that supports the entire gel. A more durable gel with a higher agarose concentration can be used for this exercise (e.g. 0.8% agarose), but the resolution of the larger DNA fragments (e.g. the 10,630 bp band from λgt10 versus the 7420 bp band from λ) is not as good.

Acknowledgments

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Simulating Genetic Change In a Large Lecture Hall

The Ultimate Bean Counting Experience

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Changes in genetic composition of populations through time is a central theme of evolutionary and ecological sciences, and result from both selective and non-selective pressures. These genetic changes through time can be dramatic or subtle. For example, a person who was not familiar with the species *Brassica oleraceae* might be astonished to learn that broccoli, Brussels sprouts, cabbage, cauliflower, kale and kohlrabi are genetic variants of this one species.

Understanding patterns of genetic variation within and between populations is extremely important to conservation biologists. Many current issues related to conservation and environmental biology, such as managing small populations of threatened or endangered species, require an understanding of population demographics and genetics. While natural selection is important, many pressures unrelated to natural selection (i.e. nonselective processes such as genetic drift, gene flow, and the founder effect) influence the level of success achieved by endangered species recovery programs. These issues can be used to capture student interest in theoretical population genetics. For example, information on captive breeding studies is available from many sources in the literature, and species such as black footed ferrets and California condors are well known to many biology students. Furthermore, understanding population genetics is crucial for rare populations in natural habitats such as grizzly bears.

Often, changes through time are measured as a change in the frequency of different variants of a particular gene, or alleles, in a population. Changes in allele frequencies for a particular trait can result from nonrandom mating, genetic drift, differential survival and reproductive success, and mutation. The Hardy-Weinberg Principle illustrates that in the absence of specific evolutionary forces, genetic variability is maintained in populations through simple Mendelian inheritance (e.g. see discussion in Raven & Johnson 1996). This principle allows calculation of genotypic frequencies in a large population with no selective forces acting on it, given no gene flow, no mutation, and random mating. Mathematical equations commonly taught in introductory biology and population biology courses illustrate the effects of violating the assumptions of this principle on genotypic frequencies.

Yet many students in introductory and upper division courses have a very difficult time understanding this mathematical model of gene frequency, as well as the implications of deviations from predictions of equilibrium states when assumptions are violated. In response to these difficulties, we developed a simulation exercise to clearly illustrate the influence of founder effects, genetic drift and gene flow. We have successfully used these simulations in large enrollment Introductory Biology courses combining majors and nonmajors (enrollment of ~450 students) and an upper division Environmental Biology course (enrollment of 25 students) using cooperative learning strategies. We also present strategies for classroom management and assessment.

A Learning Cycle Lecture on Genetic Change

The objectives for this instructional unit were to:

1. Develop student understanding of allele frequencies in a population and the influences of gene flow and genetic drift.
2. Illustrate gene flow and how it takes place
3. Collect data to illustrate the effect of genetic drift on allele frequency in small populations.

These topics were presented in a learning cycle format (e.g. Allard & Barman 1994) that promoted cooperative group work and collaboration. We adapted the approach of Ebert-May et al. (1997) for implementing cooperative group work in the large lecture hall. The activities described below required two 50-minute lecture periods to complete.

The modified learning cycle lecture on genetic change revolved around a series of related questions which moved from familiar ideas to new conceptual material related to genetic change (see also Brewer & Ebert-May, 1998). We catalyzed learning with guiding questions and opportunities for investigation, so that there was a balance between direct instruction and student interaction. Throughout the instructional period, students were provided with time to think (reflect). Moreover, we used frequent informal assessment to learn of and correct misconceptions. In this particular unit, our modified lecture was designed around *engagement, exploration/explanation, extension* and *quiz* questions.

To *engage* their interest, students were asked the following question:

> *During the last 25 years, biologists have attempted many times to help endangered species recover from the brink of extinction (e.g. trumpeter swans, California condors, black-footed ferrets). From a biological perspective, why is recovery of endangered species such a challenge?*

This question set the theme for the lecture, and drew on student background knowledge, interest and personal beliefs. Moreover, as students presented their answers to the class, it was possible for us to determine the extent to which students related

knowledge of genetic change through time to the problem. After discussing responses to this question, students were presented with a short lecture on important background concepts related to gene flow.

After the short lecture, students were asked to *explore* the concept of gene flow by answering the following question:

> *How does gene frequency change in two populations over the course of 10 generations when gene flow is occurring?*

Using the simulation procedure described in Table 1A, students worked in collaborative groups to collect data to answer these questions. This phase of the lecture built upon the previous lecture segment, and provided practice in data collection and interpretation. Students discovered that when divergence has occurred between two populations, gene flow can have a homogenizing effect.

To *elaborate* on their developing understanding of genetic change, students were asked:

> *What is the nature of change in allele frequencies in small populations?*

Using the simulation procedures described in Tables 1B and 1C, students discovered principles of the founder effect and genetic drift. The founder effect can be an important evolutionary force in populations that are established in distant or isolated places from the parent population. When founders have only a small complement of the genetic variation found in the parent population, or they contain allele frequencies that differ markedly from the parent population, allele frequencies can rapidly diverge from the original parent population. Genetic drift is the chance fluctuation in the proportion of alleles in a population due to random sampling of gametes. Genetic drift is similar to the founder effect in that a small sample from a population of gametes may not be representative of the entire pool for gametes. Like the founder effect, genetic drift is important in small populations, but not large populations.

To assess learning at the end of the learning cycle lecture and experimental simulations, students were presented with the following *quiz* question:

> *Reconsider the problem of recovering endangered species. From a genetic point of view, why are recovery efforts such a challenge?*

Students worked on an answer alone first, then they collaborated with their group to refine their answers. Before leaving, students placed their answers in boxes provided at the doors to the lecture hall. Both group and individual answers were collected for evaluation.

Preparing for the Genetic Change Simulations in the Lecture Hall

The key to successfully implementing simulations in large lectures is to have all material carefully prepared before class, and to have a plan for clearly giving instructions and distributing experimental material (e.g. Table 2). Simulation materials were prepared before class using dry beans of similar sizes but different colors (e.g. black beans and navy beans, or kidney beans and pinto beans) to represent alleles. Two kinds of "populations" of 25 individuals were established. The first population had 25 black and 25 white (navy) beans, and the second population had 40 black and 10 white beans. Each student team was provided with one of each population in an "experimental kit."

For the large lecture, 160 experimental kits were constructed. Each kit contained four different bags. To begin, Ziploc™-type bags were labeled with one of three different colors of tape. Bags with blue tape and bags with white tape received 25 white beans and 25 black beans. Bags with red tape received 40 black beans and 10 white beans. A fourth bag (no colored tape label) had no beans. Bags with red and blue tape were used in the experimental simulations while beans in the bag with white tape were used to replace beans that "escaped" during the simulation. An instruction sheet was prepared (Tables 1A–1C, 3) with learning cycle questions and experimental procedures. Finally red, blue, and white bags were bound together with the instruction sheet and placed in the empty bag to form a kit for distribution in class.

At the beginning of the class, students were instructed to form cooperative groups of three people. Students were well prepared to work in small groups because at least once *each* lecture throughout the semester students worked together in class using the methods outlined in Table 2 and recommendations in Johnson et al. (1991). In each group, one student was designated as the official bean counter (data collector), another was the materials collector and assisted with recording data, and the third was the perpetrator of the evolutionary force (manipulated beans between experimental populations). To facilitate rapid distribution of experimental kits in the large lecture hall, student volunteers provided a kit to every person (materials collector) waving his/her hand in the air (< 3

Table 1A. Simulating gene flow: evaluating the change in allele frequency due to gene flow in two populations.

Step	*Procedure*
1	Verify the number of beans in the bags with blue and red tape. Blue—25 black beans and 25 white beans Red—40 black beans and 10 white beans Replace beans from the white population if beans have been lost.
2	Use data table to record "gene frequencies" in the red and blue populations after each gene flow event for 10 generations.
3	Counter 1 closes eyes and then selects 6 beans from red bag. Counter closes eyes and selects 6 beans from blue bag. With eyes open, trade selected beans with the other bean counter in your group.
4	Drop the beans in your bag, and then count the resulting number of black and white beans (do this by looking through the bag to avoid losing a bunch of beans).
5	Record the new bean frequencies on the group data table.
6	Repeat Steps 3–5 until you have data for 10 generations.
7	Restore the number of black and white beans to their original frequency as shown in Step 1 above.
8	Answer question: *Does gene frequency change in two populations over the course of 10 generations when gene flow is occurring? Describe the nature of the changes observed in your populations.*

Table 1B. Simulating founder effect.

Step	*Procedure*
1	Verify the number of beans in the bags with blue and red tape. Replace beans from the white population if beans have been lost. Blue—25 black beans and 25 white beans Red—40 black beans and 10 white beans
2	Use data table to record allele frequencies in populations founded with members of the red population.
3	With eyes closed, select 6 beans from the red bag (represents alleles present in 3 individuals).
4	Count and record the resulting number of black and white beans selected in the founding population on the group data table.
5	Record the bean frequencies of parent population on the group data table; return beans to red bag.
6	Repeat Steps 3–5 until you have data for 10 founding events.
7	Repeat Steps 2–5 for the "blue" population.
8	Restore the number of black and white beans to their original frequency as described in Step 1 above.
9	Answer question: *Describe the patterns of allele frequencies in founding populations relative to parent populations.*

Table 1C. Simulating genetic drift.

Step	*Procedure*
1	Verify the number of beans in the bags with blue and red tape. Replace beans from the white population if beans have been lost. Blue—25 white beans and 25 black beans Red—40 black beans and 10 white beans
2	Trade a red or a blue bag with a neighboring group so that one group has two red bags and the other group has two blue bags. Both of your populations should now have the same gene frequencies. One bag will be the female gametes, and the second bag will be the male gametes.
3	With eyes closed, select 1 bean from each bag and place them in the empty bag provided in your kit. Using replacement beans from the white bag, restore the number of beans in the male and female gamete pools to their original frequencies.
4	Repeat Steps 3–4 until you have 25 new offspring (i.e. 50 beans).
5	Use data table to record "gene frequencies" in second generation.
6	Restore the number of black and white beans to their original frequency as shown in Step 1 above
7	Answer question: *What is the nature of change in allele frequencies in small populations?*

minutes). Then students were asked to verify the initial counts of beans in the bags.

Implementing Simulations in the Lecture Hall

Before beginning the simulations, procedures were described (Tables 1A–1C). For each type of simulation, students were asked to record their data in a table (e.g. Table 3).

Gene Flow Simulation

Students simulated migration or mating between populations by moving beans between populations according to the instructions in Table 1A. Either the exchange of "individuals" (pairs of beans/gametes) or mating between populations (single bean/gamete) can be simulated. In Table 3A, three individuals (6 beans) are exchanged to simulate migration between populations. Within their cooperative groups, students randomly select six beans from each population and exchange them with each other. Population allele frequency was recorded in the data table, and the migration of individuals or alleles is repeated for 10 generations. At the end of each 10-generation simulation, students were asked to restore their populations to their original allele frequencies.

Founder Effect Simulation

The class was divided into two groups (Table 3B). Half the groups worked with one parent population (25 black/25 white beans) and the other half worked with the second parent population (40 black/10 white beans). Students were asked to draw six beans from their populations, representing three individuals. The allele frequency of the original population was recorded along with the allele frequency of the new founder population. Beans in the parent population were replaced to simulate a large parent population, and the procedure was repeated 10 times, providing data for 10 independent founding events. In the class discussion, allele frequencies for the founding population simulated by each half of the class were compared to illustrate the influence of gene frequencies in the parent population on gene frequencies in the founder population.

Genetic Drift Simulation

To simulate genetic drift, populations of beans were divided into male gamete pools and female gamete pools so that allele frequency mirrored that of the entire population (Table 1C). Students were asked to exchange a bag of beans with a neighboring group so that each group had two bags with the same bean frequencies (i.e., two bags with 25 black/25 white beans in each or two bags with 40 black/10 white beans in each). A new generation was established by randomly selecting one bean from each of the gamete pools. After each selection, the gamete pool was restored to its original frequency using beans from the white bag. Gamete selection continued until 25 offspring had been formed (50 beans). The resulting gene frequency in the "second generation" was recorded, and the entire process was repeated for 10 generations. Results were

Table 2. Management strategies to organize cooperative groups (modified from Johnson et al. 1991 and Ebert-May et al. 1997).

Considerations	*Strategies/Instructions for Management*
Instructions to Students:	1. At signal, form a small group with two people sitting next to you and introduce yourselves. 2. Work together on the problem. 3. Come to a consensus on a best answer together as a group. Make sure everyone knows the answer because students will be chosen at random to report to the class. 4. When your group is finished, quietly compare answers with the surrounding groups. 5. Quickly redirect your attention to the front of the room when the signal is given (e.g. dimmed lights).
Expectations for Group Work:	1. Everyone participates. 2. Individual accountability—a random numbers table will be used to select people from the class list to share their group's answer with the class. 3. Participation is noted on the roster on the grade sheet and taken into account at the end of the semester if you are close to a grade cutoff point.
Other Management Issues:	
Student reservations about speaking in front of hundreds of peers	Verbal signal to "pass" (e.g. I'd like to think about that further); respect student ideas and responses.
Sheer volume of hundreds of conversations	Ask students to speak in "restaurant voices".
Fixed theater-style seating	Allow students to get up and move around to form groups.
Accountability	Group quizzes; "participation points" for contributing to discussion; use random numbers generator to select names off class roster and sample with replacement.

Table 3. Example data table for simulation experiments.

"Red" Population		
Generation	White Beans	Black Beans
initial count	10 beans	40 beans
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

summarized on graphs prepared by the students.

Sharing the Simulation Results

After completing each simulation, students shared results with the class. Because it is impossible to share results of 150 groups (or even 10 groups in a smaller class), answers were shared by students randomly chosen (using a random numbers generator) from the class roster. Thus, it was important for every person in the group to be able to answer the questions. When students were not ready to share their answer, they responded "I'd like to think about that further" and another student was selected.

During the simulations on gene flow, students found that gene frequencies approached equilibrium between the two populations after about five generations. For the founder effect, students found a 2:4 or 3:3 allele frequency using the mother population with equal numbers of black and white beans, but either all black alleles or 5 black:1 white ratios when they used the parent population with 40 black:10 white beans. Most students readily predicted that rare alleles may be lost if individuals with those alleles do not reproduce. Furthermore, they surmised that even if alleles are rare, they can have a particularly important influence on populations founded by just a few individuals.

Integrating an Open-Ended Investigation

To extend the simulations to a more open-ended inquiry, students were asked to consider the following question:

> *How does gene flow affect allele frequencies in large versus small populations? Design your own experimental simulation to answer this question.*

In our classes, students were encouraged to discuss the question and their approach with other groups. Many groups worked alone on this problem while other groups worked together to develop and implement a simulation. For assessment, each group was asked to submit a description of their simulation procedure, a data table, and an answer to the question.

Conclusion

A conceptual understanding of how populations change through time is important for understanding the

processes of evolution and extinction. For example, such knowledge is essential for understanding classic examples of evolutionary change (e.g. H.B.D. Kettlewell and the Peppered Moths in Hagen et al. 1996) as well as issues related to managing populations of endangered species which typically are encumbered by small population sizes. The learning cycle questions and simulations described here can set the stage for interesting, dynamic discussions related to conservation biology, genetic engineering (Brewer & Ebert-May 1998), the state of agriculture and animal husbandry for future food needs of humans, and natural selection and evolution.

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Plants

How Can Plants Tell Which Way Is Up?

Laboratory Exercises To Introduce Gravitropism

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Many people think of plants as essentially sessile organisms that do not actively respond to their environment. What could be further from the truth! In fact, plants are capable of a variety of movements, including the dramatic nastic responses (such as Venus fly trap closure) and the less sensational tropisms. These latter movements are directed growth responses to some type of external stimulus such as gravity (gravitropism, formerly known as geotropism) or light (phototropism). This paper describes some interesting exercises that are derived from recent work, including research that has led to experiments performed on two Space Shuttle missions in 1997 (Kiss et al. 1998).

The study of tropisms can be a useful way to introduce students to plant biology in high school and introductory college courses. In our experience, students are fascinated by plant movements when they are presented in lectures and find laboratory experiences on this topic quite engaging. Laboratory work on plant tropisms can also be used to introduce important concepts in science such as hypothesis testing, quantitative analysis, and the use of statistics.

The laboratory exercises described in this paper involve the higher plant *Arabidopsis thaliana*, which has become an important organism in molecular biology research and is the focus of an international plant genome project. Based on the material presented here, a number of plant gravitropism laboratory exercises with *Arabidopsis* that are simple in terms of equipment/materials and procedures can be developed. These exercises are robust in that they work well even in the hands of introductory students, and they can be expanded according to the individual instructor's needs. This paper describes two exercises that have been performed by beginning college students, and these exercises can easily be performed in biology classes in most high school settings.

Background

Gravitropism in plants can be divided into three temporal stages: perception, transduction/transmission, and response (i.e. the differential growth in a plant that leads to downward or upward curvature). These laboratory exercises are focused on the early events of plant gravitropism termed "gravity perception." Gravitropism and other tropisms in plants have been extensively studied since late in the last century, and Charles Darwin and his son Francis published a book titled *The Power of Movements in Plants* in 1883. (In fact, the Darwins' early experiments in phototropism are outlined in several introductory biology textbooks.)

Gravity perception in plants is hypothesized to be mediated by the interaction of dense organelles (called statoliths) with other cytoplasmic structures. In higher plants, a great deal of evidence suggests that amyloplasts (starch-filled plastids), which settle or sediment to the new lower cell wall following reorientation, function as statoliths. Thus, the idea to explain gravity perception in plants has been referred to as the "starch statolith hypothesis," and the evidence supporting this hypothesis has been reviewed in a number of publications (e.g. Sack 1997), including a very accessible article in *Scientific American* (Evans et al. 1986).

Some of the strongest evidence that amyloplasts function as statoliths comes from research with mutants of *Arabidopsis* that lack starch (Kiss et al. 1997). These mutants are starchless since they are missing one of the enzymes (phosphoglucomutase or pgm) in the final steps of starch synthesis. Several studies have shown that the starchless mutants are much less sensitive to gravity, but these mutants still do respond to gravity (i.e. they are not agravitropic). The upshot of this work is that plastids (with starch in the case of the normal wild-type, WT, and without starch in the case of the mutant) can function in gravity perception. (It is important to note that both the WT and mutant plants have the same number of plastids, but the mutant has starchless plastids.) However, when starch *is* present, there is a greater plastid density and an increased total mass of these statoliths, and the plants do better in terms of gravity perception and response.

In the two related laboratory exercises that follow, students should be able to demonstrate that while both the WT and mutant respond to gravity, the WT has a greater response. One exercise focuses on gravitropism in the flower stalks of mature plants, and the other involves a study of gravitropism in young seedlings. They can be performed independently or together, depending on the instructor and his or her course.

Exercise #1—Flower Stalks from Mature Plants

Materials Needed

Seeds of normal WT *Arabidopsis* and a starchless mutant are available free of charge (or in some cases, for a small fee) to educators through the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University. Orders may be placed through the Internet (http://aims.cps.msu.edu/aims/), by e-mail (arabidopsis+@osu.edu), or by standard mail (ABRC, 309 B & Z Building, 1735 Neil Ave., Columbus, OH 43210; telephone: 614-292-9371; fax: 614-292-0603). The starchless mutant is pgm-1 (stock no. CS210), and the WT strain to use is Columbia (Col-2; stock number CS907). The ABRC will not send many seeds, but you can grow more plants for your own seed stock (i.e. plants self-pollinate), and each plant will produce thousands of

seeds. Other materials needed for this exercise include soil, pots, a light bank (fluorescent "shop" lights work well), a light-tight box (e.g. photocopy paper boxes or large shoe boxes), and protractors for measuring angles.

Preparation

You will need to grow plants in pots until they start forming flower stalks. There are many ways to do this, but we will provide highlights of one general method here, and the reader is referred to the above ABRC site to learn more details (if needed) and alternatives in growing these plants. The good news is that *Arabidopsis* can be grown in a variety of containers and in many ways, including in growth chambers, greenhouses, on window ledges, and outdoors (after all, it is a real weed).

The first thing to realize is that *Arabidopsis* seeds are very small (0.4 mm in diameter), and one has to be careful in handling them. About one to two dozen seeds are sown by sprinkling them in four-inch plastic pots in a commercial potting soil such as Metromix 350 or Peter's potting soil. Seeds should not be covered with additional soil because they require light for germination. Several pots can be placed in larger plastic containers that can be covered with a plastic wrap in order to provide a humid atmosphere to stimulate germination. At this point, the pots are placed under continuous illumination from 40-watt fluorescent bulbs in a "shop" light fixture. Remarkably, the starchless mutant and WT are identical in morphology and size if they are maintained together under continuous illumination. If plants are grown on a light/dark cycle, then the mutant may be smaller and develop at a slower rate compared to the WT. However, we have found that the growth rate, in continuous light, of the WT and mutant flower stalks is almost identical; thus potential differences in growth are not significant when evaluating gravitropism.

An alternate to growing the plants in a soil mixture is to use a synthetic substrate, such as Rockwool cubes or Oasis floral foam cubes, purchased from a greenhouse or gardening supply store. The advantage of this method is that there is no potential problem with soil spilling when the plants are reoriented.

In any case, soil moisture should be carefully maintained until the seeds germinate, which should be in about three to five days after sowing. Under conditions of low humidity, it may take a few more days for germination. Seeds will germinate at room temperature (20 to 22° C), but temperatures above 25° C should be avoided. Following germination, plants should be watered as needed, but too much watering may lead to fungal growth. *Arabidopsis* is in the mustard family, and the plants will form rosettes of leaves. Once the plants are at the rosette stage, they may need to be thinned to avoid overcrowding in the pots. About three to four weeks after sowing of seeds, the rosettes will start to form flower stalks or inflorescences. The gravitropism experiments work best on flower stalks that are 2 to 6 cm in length.

Procedures

After the above preparation, the experiment itself is quite simple. Both WT and mutant plants are reoriented 90 degrees in the dark, and this beginning of the experiment is referred to as time 0. We use photocopy paper boxes for the dark environment, and reoriented plants are carefully removed from the boxes at several time intervals (e.g. 0.5 hour, 1.0 hour, 2.0 hours, 3.0 hours). Do not worry about the time the plants are exposed to light since the phototropic response is relatively weak. (Instructors can excise flower stalks at the end of the experiment, and if the plants are left in favorable conditions, they will continue to produce additional stalks that can be used for other experiments.)

Curvature is measured at each time interval with a protractor, and the plants then are returned to the box. Sample data are shown in Table 1. Students can observe that WT flower stalks curve faster compared to the mutant flower stalks, even though the growth rates of both strains are similar. The data can be presented in table format or values can be used to draw a graph. Instructors can also have the students perform some simple statistical analyses (such as a *t*-test) to determine if the curvature values are significantly different between the WT and the mutant flower stalks.

If a light-tight box is not practical to implement, it is possible to do this experiment in general room light since the phototropic response is much weaker than the gravitropic response in these flower stalks. Another variation is simply to do the experiment with the WT only, and this exercise still allows students to appreciate the phenomenon in plants and to do some data analysis. The upward curvature of WT flower stalks is illustrated in Figure 1.

This exercise can be enhanced by demonstrating the differing phenotypes of the WT and starchless mutant. Leaves from the plants are excised and left overnight in ethanol to remove the chlorophyll. The leaves then are placed in an iodine solution (i.e. 2.0 g potassium iodide and 0.2 g iodine are added to 100 mL of water), and the WT will stain intensely black while there will be no reaction in the starchless mutant.

Exercise #2—Young Seedlings

Materials Needed

Seeds of normal WT *Arabidopsis* and a starchless mutant are available through the ABRC as indicated above. Other materials needed include: agar, disposable petri dishes (9 cm diameter), Parafilm™, commercial bleach, disposable Pasteur pipets, small vials, fluorescent "shop" lights, aluminum foil, and protractors.

Preparation

An agar mixture, 1.0 to 1.2% (w/v), is autoclaved and prepared by standard microbiological methods. At the same time, some water is autoclaved (or sterile water can be purchased). The agar solution is poured into petri dishes, which are filled to about one third of their height. The petri dishes then are left to cool until the agar solidifies.

Prepared agar plates or ready-to-pour agar that only needs to be heated in a microwave oven can be purchased (e.g. Wards Natural Science Inc., Box 92912, Rochester, NY 14692-9012) if an autoclave is not available. A nutrient agar can also be used and is preferable; recipes can be found at the ARBC Web site listed above or in Kiss et al. (1996).

Table 1. Mean upward curvature in degrees (± standard error) of *Arabidopsis* flower stalks following a 90-degree reorientation in the dark. Curvature in the normal wild-type is compared to that of a starchless mutant ($97 < n < 100$).

Time after reorientation (hours)	*(WT)*	*(Mutant)*
0.5	7.3 ± 1.3	2.8 ± 1.1
1.0	40.6 ± 2.7	24.9 ± 1.5
2.0	78.4 ± 3.5	48.8 ± 1.9
3.0	92.2 ± 3.1	62.9 ± 2.4

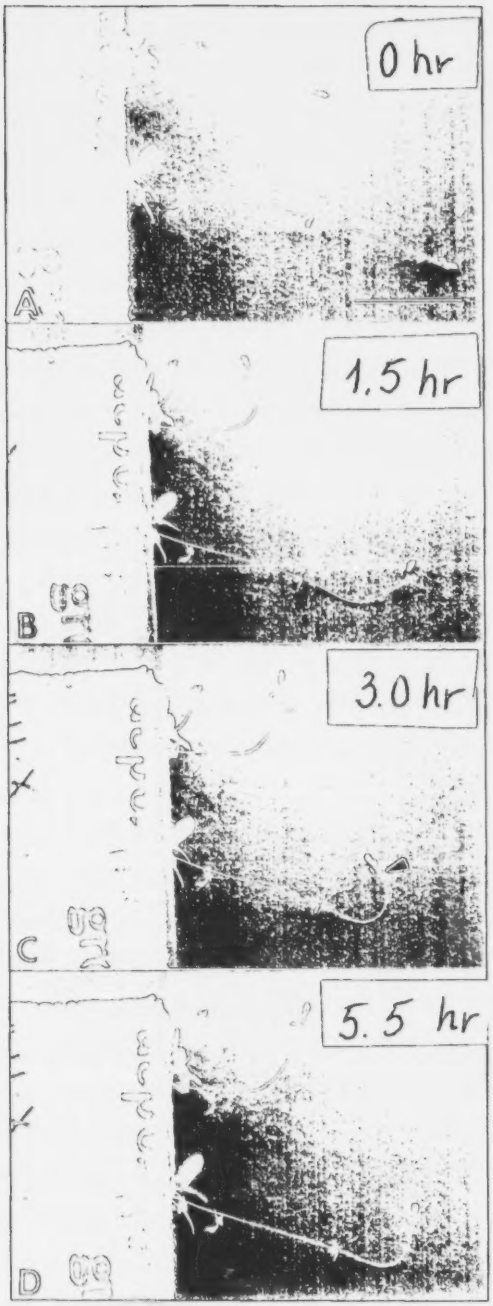

Figure 1. Gravitropic curvature in normal wild-type *Arabidopsis* flower stalks. At time 0 (A), plants were reoriented 90 degrees, and photographs were taken at 1.5 hours (B), 3.0 hours (C), and 5.5 hours (D) following reorientation. The gravity vector is toward the bottom of the photographs. Upward curvature is rapid, and some flower stalks (arrowhead in panel C) "over-shoot" the gravity vector and then "correct" themselves (panel D). Scale bar = 2 cm.

Arabidopsis seeds are surface sterilized in a 30% (v/v) solution of Clorox® for 15 minutes in a vial, and then are rinsed four times (10 minutes for each step) in sterile water. It helps to place a drop of a detergent such as Triton X-100 or Tween (or even dishwashing liquid) into the Clorox solution and rinse water. Seeds are placed onto the surface of the agar with a Pasteur pipet and are placed approximately 1 cm apart. In the petri dishes, two rows of seeds can be accommodated. Petri dishes are sealed with Parafilm™ and placed on edge in some type of rack so that the surface of the agar is vertical, and an arrow is drawn on the dish to indicate the direction of gravity (Figure 2). At this point, people walking into your lab will realize something new (and maybe strange) is happening since petri dishes are typically placed flat on the bench!

The dishes are placed under a fluorescent "shop" light for 24 hours. Even though this is a "dark-grown" experiment, the seeds have a light requirement for germination. After the 24-hour light treatment, the dishes are wrapped in aluminum foil and placed in a dark environment such as a shoe box or in a drawer. It also helps to make an arrow on the foil as well as the petri dish itself to indicate the direction of gravity. Seedlings that are 1.0 to 1.5 cm in length should develop in about three days following the wrapping of the petri dishes with foil.

Procedures

Petri dishes with four-day-old seedlings are removed from the dark, and the aluminum foil is unwrapped. Students should easily see that the WT seedlings are oriented relative to gravity while the mutant seedlings are disoriented (Figure 3). This deviation can be quantified by measuring with a protractor the angles that hypocotyls are deviated from the vertical gravity vector. If the vertical gravity vector is defined as 0 degrees, the mean angle for the WT should be about 0 degrees, and the mean angle for the mutant should be about 60 degrees. Orientation of the roots can also be examined.

To extend this exercise, students can perform a time course of curvature

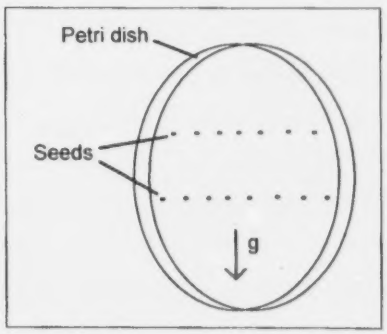

Figure 2. Diagram of petri dish setup used in experiments to study gravitropism in young seedlings. The arrow indicates the gravity vector.

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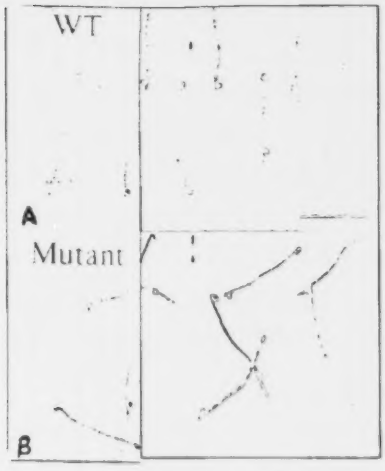

Figure 3. Orientation of 4-day-old, dark-grown *Arabidopsis* seedlings in petri dishes. The gravity vector is toward the bottom of the photographs. Note that the wild-type (WT) is oriented to gravity (A) while the starchless mutant is relatively disoriented (B). Scale bar = 5 cm.

study of the hypocotyls as is outlined above in the flower stalk experiment. Petri dishes with seedlings are reoriented 90 degrees in the dark. The response to reorientation is much slower than is observed in the flower stalks, so time points for observation might include 1 hour, 3 hours, 8 hours, and 24 hours. Seedlings are removed from the dark at these time intervals, and curvature of the hypocotyl is measured with a protractor. Sample data for hypocotyl curvature is shown in Table 2, but the same seedlings can also be used for measurements of curvature of the roots.

Students should be able to see that the mutant does respond to the reorientation but at a much slower rate than the WT. (Remember, since growth rates of the WT and mutant seedlings are nearly identical, growth is not a limiting factor for the mutant.) An important point is that the mutant is not agravitropic as might be the initial hypothesis based on the qualitative observations of the orientation experiment (i.e. the mutant is severely disoriented).

Another simple extension of this exercise is to stain the seedlings with iodine solution (described above) and then observe them with a compound light microscope. The difference between the WT and mutant is quite dramatic as is illustrated by the root cap starch in Figure 4, and the difference in the shoots of both strains can also be examined.

Table 2. Mean upward curvature in degrees (± standard error) of the stem-like hypocotyls of *Arabidopsis* following a 90-degree reorientation in the dark. Curvature in the normal wild-type is compared to that of a starchless mutant ($94 < n < 251$).

Time after reorientation (hours)	*WT*	*Mutant*
1	33.0 ± 1.0	1.8 ± 1.1
3	37.6 ± 1.2	1.8 ± 0.9
8	52.3 ± 1.1	6.4 ± 1.2
24	57.7 ± 0.9	13.5 ± 1.4

Supplementary Resources

The best way to convey the excitement of the study of plant movements is by illustrating the process with motion pictures. To this end, an excellent Internet resource with time lapse movies of tropisms and other plant movements has been developed by Dr. Roger Hangarter of Indiana University (http://sunflower.bio.indiana.edu./rhangart/plantmotion/). These movies clearly demonstrate the dramatic nature of various plant movements and feature *Arabidopsis* as well as other plants. The topics include seed germination, gravitropism, phototropism, "sleep movements," nutation, and flower opening. Particularly relevant to the laboratory exercises described in this paper is the fact that gravitropism in *Arabidopsis* flower stalks is part of the movie collection.

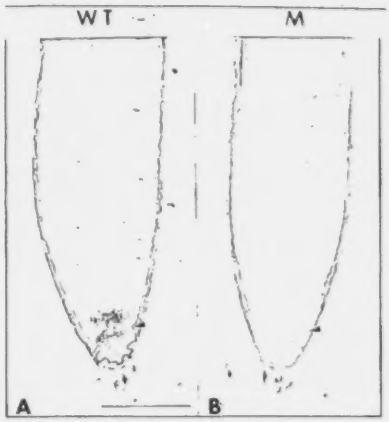

Figure 4. Roots of *Arabidopsis* seedlings stained with iodine solution. Note the intense, dark reaction (which indicates starch) in the root cap (arrowhead) of the wild-type (WT) in panel A while there is no reaction in the root cap (arrowhead) of the starchless mutant (M) in panel B. Scale bar = 100 μm.

Another useful supplement to these laboratories is a two-page cartoon in the August 1997 issue of *Discover* magazine. Plant gravitropism is introduced in a humorous, engaging (yet accurate) manner in a cartoon titled "Starch wars" (Gonick 1997), which has been used in an educational outreach program for high school teachers by North Carolina State University.

Conclusions & Perspectives

In terms of scientific conclusions, the important point is that while both the WT and mutant respond to gravity, the response (i.e. curvature) is greater and more rapid in the WT. Based on their data, students can make arguments for and against the starch statolith theory for plant gravity perception. While it is clear that starch *per se* is not needed for gravitropism, a full complement of starch (as in the WT) provides a better gravitropic response. Thus, the results are still consistent with the essence of a statolith model (i.e. dense particles participating in gravity perception). Perhaps the idea should be called the "plastid statolith hypothesis" since it appears that plastids (with starch in the case of the WT, and without starch in the case of the mutant) can function in gravity perception. Instructors will find a more detailed discussion of these ideas in Salisbury (1993) and Sack (1997).

From the perspective of teaching this material, we have found that students in introductory college biology courses enjoy the laboratory exercises outlined in this article. They like the idea of performing experiments that are based on current research and the relationship of the exercises to the space program. After all, space has been and continues to be a useful way to promote and encourage interest in the natural sciences. Students also value the opportunity to gather "real" data that can be used to evaluate a scientific hypothesis.

While we do not have experience with these experiments in a high school setting, they seem to be appropriate and quite feasible for biology classes at the secondary level. The two exercises are very flexible in that they can be performed independently or coupled together as part of a larger project. They also can be performed with fairly basic equipment and entirely on a macroscopic scale if needed. Appropriate add-on portions for each exercise have been discussed

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for the instructor who has the need and time for more complex laboratory experiences. Finally, we believe that these laboratory exercises will stimulate students to think carefully about their results and to develop critical thinking skills that will be useful to them throughout their educational careers.

Acknowledgments

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Thigmomorphogenesis in the Classroom

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Thigmomorphogenesis is the process by which plants, when subjected to mechanical stimulation, tend to be shorter and thicker than plants that are not physically stimulated (Salisbury & Ross 1985). Several studies have shown that many different kinds of plants exhibit thigmomorphogenesis (Jaffe 1973; Mitchell 1975). In 1980, Jaffe showed that even plants that were exposed to wind exhibited thigmomorphogenesis.

I wanted to see if Wisconsin Fast Plants (*Brassica rapa*) would show a thigmomorphogenic response when exposed to a breeze. I also wanted to see if this response could be produced and studied in a classroom setting, using items that are usually found in a science classroom or are readily available at a reasonable price.

While many classes study plant growth, not many study thigmomorphogenesis. I thought that teachers and students would be interested in studying a type of plant response that is somewhat out of the ordinary.

I chose to work with fast plants because they grow and develop very rapidly (approximately 30 days from seed to seed). This allows the whole experiment to be completed in one month or less, depending on how you choose to structure it.

Materials & Methods

Plant Material

Seeds of the plant *Brassica rapa* (Wisconsin Fast Plants) were grown in a commercial potting soil, with the trade name Pro-mix.™

Environment

The plants were grown under classroom conditions in the Fogarty Life Science Building at Rhode Island College. The average temperature during the first trial ranged from 23–29° C. During the second trial, the average temperature ranged from 20–25° C. The relative humidity varied greatly, as the weather and time of year changed.

Experimental Design

The plants were planted in three standard disposable nine-cell starter flats, such as the kind used to grow seedlings in commercial greenhouses. Each cell measured approximately 2 cm × 2 cm and was approximately 4 cm deep. Four seeds were planted in each cell by placing them on the surface of the soil and covering them with 1/4 of an inch of soil. Once the plants had grown to approximately 2 cm, they were thinned out so that each cell contained one plant.

The plants were watered using a wick system. First, cotton string was cut into 10-cm lengths, two pieces of string for each cell. Then the string was inserted into the bottom of the cell and pulled up until it was about 1 cm from the top. The cell was then filled with soil.

These flats were placed in a tray of standing water for 24 hours. When the soil and string were saturated, the flats were placed onto test tube racks. This allowed the string to be threaded through the holes in the rack. The test tube racks were then placed into a dissecting tray which contained distilled water. This allowed the string, which had been put into the flats, to run through the racks and into the water. As the water in the flats either evaporated or was used by the plants, the string would act as a wick drawing water from the tray into the soil. In this manner, the plants could be watered without being touched.

Since the Fast Plants require intense light, I used a moveable light source. Two 4' shop lights, with grow-lux bulbs, were suspended from a pulley that was attached to a pipe that ran across the classroom. This allowed me to maintain a continuous light source at a constant distance of approximately 2 cm from the tops of the plants (University of Wisconsin 1989).

One flat was used as a control. The other two flats were labeled as Experiment 1 and 2 respectively. While the controls were allowed to grow without being disturbed, the experimental plants were not. The experimental plants were exposed to a wind that was generated by an ordinary 18" house fan.

For the first trial, the Experimental #1 group was exposed to the wind constantly, while the Experimental #2 group was exposed for one hour every day. For the second trial, both experimental groups were exposed for one hour every day. Both trials ran for 30 days.

Growth was measured by the total height, diameter, and dry weights of the plants.

Results

In both trials the results were very noticeable (Tables 1, 2 & 3). The group that was exposed to the wind for one hour seemed to exhibit a reduction in height and an increase in width. They were approximately 18% shorter and 30% thicker than the experimental plants. Meanwhile, there was less than 10% difference in their dry weights.

During the second trial I noticed that all of the plants (both the experimentals and the controls) were smaller than in the first trial. I would attribute

Table 1. Differences in plant growth due to exposure to a breeze for one hour daily for 30 days.

		height (cm)	*% shorter than control*	*diameter (cm)*	*% thicker than control*	*dry weight (g)*
TRIAL 1	control	35		0.2		0.34
	experiment	29	18	0.27	35	0.31
TRIAL 2	control	28		0.17		0.23
	experiment	18	37	0.23	35	0.22

Table 2. Raw data from first trial of plant responses to a breeze lasting one hour daily for 30 days.

	plant #	*height (cm)*	*diameter (cm)*	*dry weight (g)*
CONTROL PLANTS	1	40	0.24	
	2	35	0.16	
	3	30	0.17	
	4	29	0.23	
	5	39	0.22	
	6	36	0.18	
	7	34	0.2	
	8	35	0.21	
	9	36	0.2	
	average	35	0.2	0.34
EXPERIMENTAL PLANTS	1	32	0.3	
	2	26	0.24	
	3	33	0.28	
	4	30	0.26	
	5	27	0.28	
	6	28	0.25	
	7	29	0.28	
	8	26	0.29	
	9	31	0.22	
	average	29	0.27	0.31

Table 3. Raw data from second trial of plant responses to a breeze lasting one hour daily for 30 days.

	plant #	*height (cm)*	*diameter (cm)*	*dry weight (g)*
CONTROL PLANTS	1	27	0.20	
	2	28	0.18	
	3	21	0.21	
	4	22	0.10	
	5	30	0.13	
	6	25	0.20	
	7	32	0.20	
	8	35	0.15	
	9	30	0.16	
	average	29	0.17	0.23
EXPERIMENTAL PLANTS	1	14	0.26	
	2	18	0.27	
	3	20	0.26	
	4	18	0.25	
	5	18	0.22	
	6	22	0.27	
	7	15	0.20	
	8	20	0.27	
	9	18	0.23	
	10	19	0.19	
	11	13	0.25	
	12	14	0.21	
	13	20	0.20	
	14	15	0.28	
	15	16	0.20	
	16	17	0.20	
	17	18	0.22	
	18	22	0.20	
	average	18	0.23	0.22

this to the fact that the room was colder than during the first trial. However, the results were the same. The experimental plants were approximately 37% shorter and 35% thicker than the control plants.

Discussion

The exposure of *Brassica rapa* to a breeze does seem to affect the plants' growth. When the plants were exposed to a breeze for one hour a day they showed responses that were similar to those reported in the literature. However, this was not the case with the first group of plants that were constantly exposed to a breeze.

The group that was constantly exposed to the wind showed a very different response. These plants were 54% shorter than the control group. But they were also 20% thinner and they had a much lower dry weight (65%). This group was also morphologically different. The windward side leaves were tiny or nonexistent while the leeward side leaves were larger. This may have been due to dehydration caused by increased transpiration.

While this response was interesting and may suggest another avenue for study, it was not the response that I was interested in. Therefore, when a second trial was run, I exposed two flats to the wind for one hour a day. This would increase the sample size to see if my results were repeatable.

My results are consistent with those of Jaffe and others. The experimental plants showed a reduction in height and an increase in width. Therefore, I feel that thigmotropism can be induced and studied in a classroom.

Suggested Classroom Applications

This basic research design offers a variety of educational activities. First, since the topic is not usually covered in most high school curricula, many students will not be aware of the concept and therefore be unable to predetermine the results. This will allow for an unbiased attempt to conduct a research project.

While this project can be altered to fit the needs of any curriculum, it lends itself to a full-fledged scientific investigation. This activity could consist of using multimedia centers, as well as the Internet, to research the topic. This may motivate some students to participate because they may

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not believe that plants can be influenced by the wind.

Once the background information was complete, students would have to design and set up their own experiment. This allows for discussion of the scientific method and the development of a system to test a hypothesis. Also, the students will need to decide what types of measurements need to be taken. This allows for a discussion of how plants grow and develop.

Since fast plants are relatively small, careful measurements are needed in order to notice results. This allows students to realize the importance of careful work in the lab.

Finally, the students can participate in a cross-curricular activity by transforming their research, data and results into a formal research paper. This may help students to realize that report writing is used in areas other than English class.

This setup provides opportunities to study things other than thigmomorphogenesis. By increasing the duration of exposure to the wind or the wind velocity, you can study other morphological responses in plants. For example, the growth patterns of plants at, and above, the tree line can be studied. As the wind is increased, the windward sides of the plants get smaller. As reported earlier in this paper, there are also many other effects that can be studied, depending on time and interest level.

If the wind is continually increased, then it can be studied as a limiting factor. Then students can design new experiments to see whether evaporation, force, or some other factor causes the plants to grow differently.

This activity provides the opportunity for students to investigate an unusual aspect of plant growth and development. With modifications this basic setup can be used in grades pre-K through 12 and beyond.

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Induction of Crown Gall on Carrot Slices

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Laboratory experiments on the transfer of a plasmid to a bacterium, whether by conjugation, transformation or transduction, are available for use in the teaching laboratory. However, the transfer of plasmid from a bacterium (prokaryote) to a plant cell (eukaryote) has received little attention. The following is an experiment for studying this latter type of genetic transformation.

The Gram negative soil bacterium, *Agrobacterium tumefaciens* L., is the causative agent of crown gall disease, a malignant tumor, occurring on the stems and leaves of infected dicotyledonous plants. Induction of this tumour is mediated by the presence in the bacterium of the large (200 kbp in length) tumor-inducing (Ti) plasmid. Upon bacterial infection of a wounded plant, phenolic defense chemicals released from the plant activate virulence genes on the Ti plasmid, leading to the processing and transferring of a small (20–23 kbp) segment of the Ti plasmid into the host plant cell. The bacterium remains outside the plant cell, living in the intercellular spaces of the plant. By transference through a conjugation tube initiated by the bacterium, this small segment of the Ti plasmid, designated transferred DNA or T-DNA, penetrates the host plant cell and subsequently incorporates into the plant's genome. T-DNA contains genes for the syntheses of opines, chemicals used as sources of carbon and nitrogen by the infecting bacterium, and phytohormones. Overproduction of these phytohormones (i.e. auxin and cytokinin) results in uncontrolled plant cell growth and hence the development of a tumor (Gelvin & Karcher 1996; Russell 1996).

In this exercise we induce crown gall on carrot slices placed in a petri dish containing agar and maintained for 10–14 days in a humidified atmosphere. Three carrot slices are used. One slice is inoculated with a broth culture of *A. tumefaciens*; crown gall will form on this slice. The two other carrot slices serve as controls. To show that crown gall formation is not due to chemical components in the broth itself, the second carrot slice is inoculated only with sterile broth. To further demonstrate that crown gall formation requires direct bacterium-plant cell contact and that it is not caused by chemicals synthesized and released by the bacterium (e.g. a carcinogen), the third carrot slice is inoculated with filtered bacterial broth medium in which *A. tumefaciens* was grown and subsequently passed through a 0.2 μm syringe filter. The pore size of this filter is small enough to prevent the passage of the bacterium. Thus, this control contains medium (termed *spent broth*) in which the bacterium grew and produced metabolites but lacks the microbe itself.

Procedure

First Session

To avoid unnecessary contamination, a knife, forceps, and vegetable peeler are kept in a vessel containing 70% alcohol. The bacterium is grown in tryptic soy broth at 26° C. Because the plasmid does not replicate above 37° C, care should be taken to avoid elevated temperatures throughout the entire experiment.

1. Wash and peel a fresh carrot, preferably one with green leaves at the top.
2. Using a sterilized knife, remove carrot ends to produce a 75-cm carrot segment. This segment should contain enough material to yield approximately six to nine carrot slices and should be sufficient for use by two to three students.
3. Place this carrot segment into a vessel containing 6.4% bleach for about 4 minutes.
4. With sterilized forceps, pick up the carrot segment and let the excess bleach drain into the vessel.
5. To remove any residual bleach, soak the carrot segment in three changes of sterile water (about 4 minutes each rinse).
6. Place the carrot segment into an empty, sterile petri dish and, using a large sterile scalpel, cut a 5-cm slice from the top and bottom of the carrot segment; discard these slices because they were directly exposed to the bleach.
7. Using the scalpel, cut the carrot segment into slices about 0.5 cm in thickness.
8. Prepare a petri dish containing agar (1.5% Bacto-agar) and, with marker pen, divide the bottom of the plate into three equal, triangular sectors. Label one sector "Bacterium," another "Fresh Broth," and the third sector label "Spent Broth."
9. With sterile forceps, transfer a carrot slice to each sector.
10. Using a micropipet, inoculate 10 μl of a broth culture of *A. tumefaciens* onto the center of the carrot slice located in the sector marked "Bacterium." Discard this tip. Using a fresh pipet tip for each subsequent inoculation, transfer similar aliquots of fresh broth and of spent broth to the appropriately labeled carrot slices.
11. Cover the petri dish and place it in a humidified chamber (e.g. a large, partially sealed canister with water on the bottom) housed in the dark within an incubator set at 26° C.

Second Session

(About 14–21 days later, depending in the strain of *A. tumefaciens*):

1. Examine the slices. The crown gall appears as a concentric ring of a creamy-to-yellowish lumpy growth surrounding the pith of the carrot slice (Figure 1).
2. Using a sterile single-edged razor or scalpel, make a longitudinal cut through the gall and the plant tissue. From this, prepare a thin slice for microscopy.
3. Transfer this thin slice to a microscope slide and view, initially, with the scanning (4X) objective and then with the low power (10X) objective. There is no need to add a coverslip.
4. For better contrast, add a few drops of methylene blue to the thin slice of carrot. After one

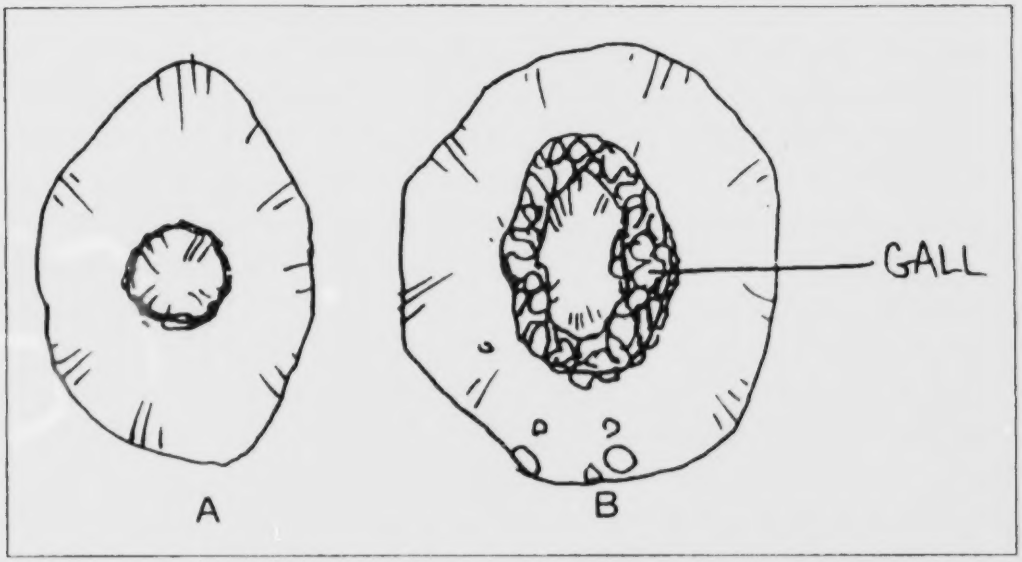

Figure 1. Crown gall formation on a carrot slice. (A) Control: inoculated only with bacterial growth medium. (B) Experimental: inoculated with a broth culture of *Agrobacterium tumefaciens*.

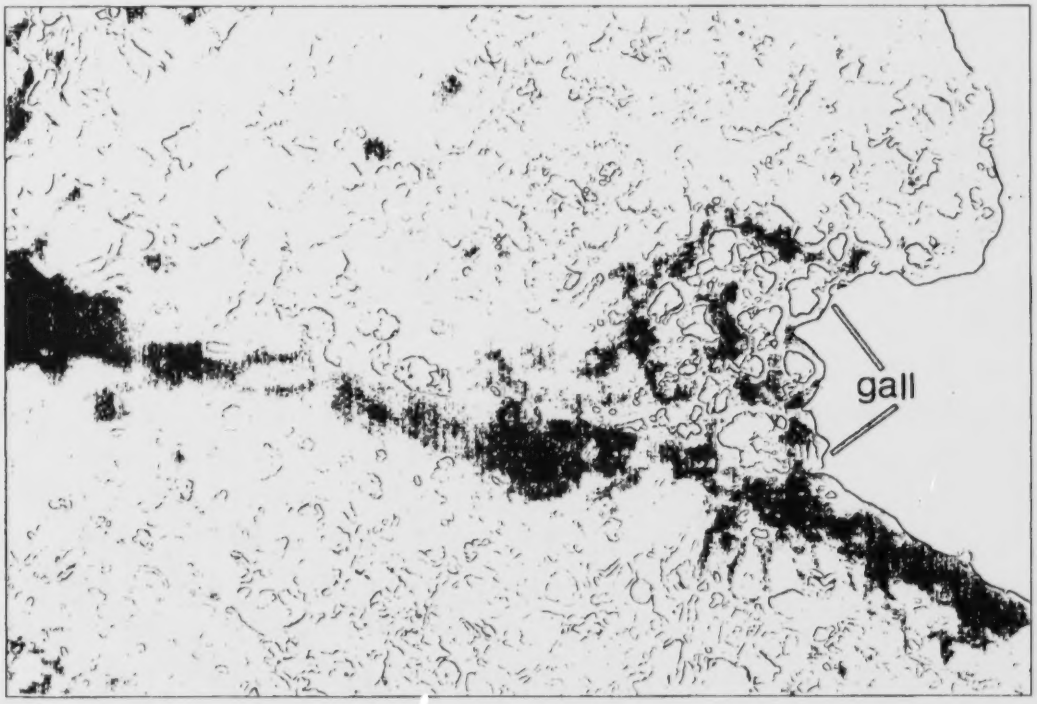

Figure 2. Microscopic appearance of cells from crown gall and from uninfected carrot tissue. Original magnification: 100X; unstained.

minute, add a few drops of water to remove the excess stain and draw off the excess fluid with an absorbent towel.

5. Observe under scanning and lower power objectives. The cells comprising the gall appear smaller and disorganized; whereas, the cells of uninfected, nonmalignant carrot tissue are organized with a definitive morphology. Xylem tissue will be noted in the uninfected carrot tissue (Figure 2).

Discussion

A meaningful discussion can be generated on the use of the Ti plasmid

to establish transgenic plants. By removing nonessential genes from the T-DNA region, especially those controlling malignancy and tumor formation, researchers have constructed genetic vectors for foreign DNA, including genes from a variety of plant species as well as animals and bacteria, that effectively transfer desirable genes to the transfected plant genome (Russell 1996).

This experiment has several applications depending on the needs of the class. The following procedure can be followed to demonstrate that the bacterium is still associated with the gall:

1. Remove some of the crown gall from the carrot slice.
2. Place it in a sterile mortar and add sterile water.
3. Mash with a sterile pestle.
4. Streak the resultant slurry on a tryptic soy agar plate.

After appearance of bacterial growth in about 24 to 48 hours, a smear can be prepared and stained for microscopic examination (Johnson & Case 1992).

To demonstrate crown gall development in the intact plant, a potted dicotyledon plant (e.g. sunflower; tomato) can be inoculated with *A. tumefaciens* (Johnson & Case 1992). To emphasize that the induction of plant cancer is due to genes on the plasmid, rather than to those on the bacterial chromosome, carrot slices or intact plants can be inoculated with a plasmidless strain of *A. tumefaciens*. [A plasmidless strain of *A. tumefaciens* (ATCC 51350) can be obtained from the American Type Culture Collection (Rockville, MD).] To add a biotechnology component to the exercise, gel electrophoresis can be used comparing the DNA isolated from plasmid and plasmidless strains of *A. tumefaciens* (Lebowitz & Sanderfoot 1991).

The experiment even in its simplest form, i.e. tumor induction on slices of carrot, is unique in that it involves gene transfer from a bacterium to a plant. Furthermore, the induction of malignancy in plants is both exciting and novel to most students. A Plant Cancer Study Kit (P7-15-4741) is available from the Carolina Biological Supply Company (Burlington, NC).

Acknowledgments

Appreciation is expressed to David J. Fox for the artwork and to Yeshiva University for providing financial support.

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Tree Measurements

An Outdoor Activity for Learning the Principles of Scaling

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The activity described in this article deals with some fundamental principles of *scaling*, which provides a mathematical basis for explaining many biological differences between small and large organisms. I have used this activity in college level courses, both in botany courses for biology majors and in introductory biology courses for general studies. Since it builds upon simple mathematical principles, it can also be used in high school biology courses. The students go outside to measure heights and trunk diameters of trees, and return to the class to analyze and discuss their results. This activity also provides experience with hypothesis-testing and elementary statistical analysis.

The Hypothesis

The principles of allometry and scaling have been extensively explored in the classic book by D'Arcy Wentworth Thompson (Bonner 1961) and more recently by Gould (1977), Peters (1983), Schmidt-Nielsen (1984), McGowan (1994), and Niklas (1994). Compare a large organism with an organism whose linear dimensions are 10 times smaller. The surface area and cross-sectional areas of support structures will be 100 times greater, and the volume 1,000 times greater, in the large organism than in the small organism. This difference has many implications for the biology of the two organisms: for example the diffusion of molecules, diffusion of heat, and strength of support structures.

In this project, the students will focus only on the strength of tree trunks. The trunk must hold up the entire aerial compression weight of the tree. The strength of the trunk in supporting aerial compression weight is proportional to its cross-sectional area. In addition, the trunk must stand up to the forces of the wind, and the strength of the root system anchors the tree, but these processes are not considered in this project.

Consider an imaginary tree that grows from 10 meters to 20 meters in height ($x = 2$). Assume that it maintains the same relative proportions of its trunk, stems and leaves as it does so (geometric similitude; Niklas 1994, p. 165). Its height and trunk diameter have increased by a factor of 2 (x). The strength of its trunk has increased by a factor of 4 (x^2), since the strength is proportional to the cross-sectional area. The aerial weight of the tree, proportional to its volume, has increased by a factor of 8 (x^3). A problem results from this: a tree 8 times as heavy must be supported by a trunk only 4 times as strong.

Obviously, real trees do not grow this way. The solution to the problem is that, as trees grow, the trunk diameter must increase relatively more than the height. This principle should apply to a comparison of trees of different sizes as well as to the growth of an individual tree. The testable hypothesis can be worded:

> *Over a range of tree sizes, trunk diameter increases disproportionately more than height.*

In fact, in a hypothetical tree that is twice as tall as another, the trunk should be at least 8 times as strong to support the 8-fold increase in weight, therefore its trunk diameter should be at least $x^{3/2} = \sqrt{8} \approx 2.8$ times as great (elastic similarity model; Niklas 1994:165). The null hypothesis is that height and trunk diameter will increase by the same proportion.

Techniques, Equipment & Procedure

Selection of Trees

The instructor should prepare a tree map beforehand, indicating the trees that each group of students will measure. To maximize ease of measurement, the instructor should choose trees that are out in the open, for example in a municipal park. The instructor should also choose trees of similar shape in order to minimize variation in data due to factors other than scaling. Trees crowded together in a woodlot are not only difficult to measure but may be taller and weaker due to competition and to being supported partly by intertwining branches. Very small saplings and trees with damage or abnormal or unusual growth (e.g. two trees fused together at the trunk), should be avoided. In general, a mixture of conifers and hardwoods should also be avoided, and trees of very different shape (for example, poplars and oaks) should not be pooled together. This is because narrower trees (such as conifers and poplars) may have less weight to support for each unit of height because they grow upward more than they grow outward. Because the principles of scaling may apply differently to large trees vs. saplings, and to conifers vs. hardwoods (Niklas 1994, pp. 167-173), the instructor should choose broad-crowned hardwood trees that have surpassed the sapling stage of growth.

However, it is not only allowable but advisable to choose trees of many different hardwood species. The instructor should choose trees of a wide range of sizes past the sapling stage, for example from 2 to at least 20 meters in height. Each student group should measure a set that contains trees of different sizes.

Measurement of Trunk Diameter

Students can measure trunk diameter with a meter tape. They measure circumference and divide by π. DBH tapes used in forestry give a direct measurement of diameter at breast height (DBH) because the centimeters are inflated by a factor of π. The students in each group record the DBH on a data sheet provided by the instructor.

Measurement of Tree Height

Students use triangulation to calculate tree height, an activity that gives them valuable review in trigonometry. Tree height above eye level (y) is the product of the distance of the observer from the tree (x) by the tangent of the angle of declination of the top of the tree ($\tan\theta$) (Figure 1). Student groups

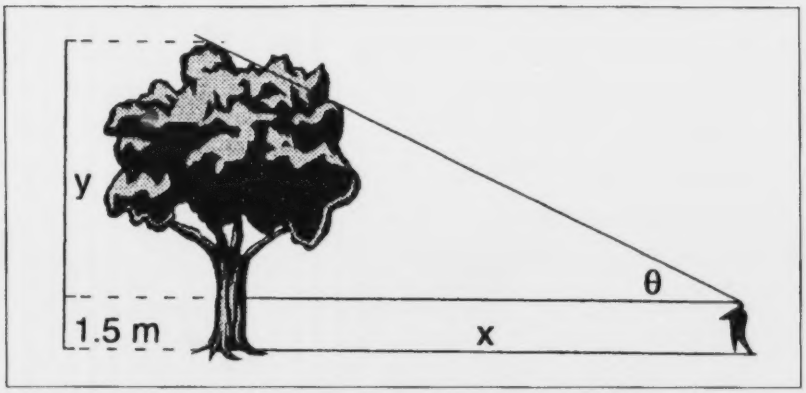

Figure 1. Trigonometric relationships among tree height, angle of declination, and the observer. (Illustration used by permission from RonJon Publishing, Inc.)

make these measurements in the following manner:

1. They use a meter tape to measure the arbitrary distance from the trunk to the observer. The observer must be far enough away to obtain a clear view of the top of the tree.
2. The observer uses a clinometer (available from forestry suppliers) to measure the angle of declination. A simple clinometer can be made from a weight, string and protractor. The students must be careful to measure degrees, not percent slope, both of which are read by the Suunto clinometer.
3. Students record x and θ in the field. In the laboratory, they calculate $\tan\theta$ and add 1.5 m, the eye level height of a typical human.

Calculation of Results

Student groups provide ordered pairs of trunk diameter and tree height measures to the instructor. What the instructor does next depends on the constraints of equipment and time.

1. The students should receive, or help to create, a visual display of their data: for example a scatter plot of tree heights as horizontal axis and trunk diameters as vertical axis (Figure 2). This can be done on graph paper, on the chalkboard, or by computer spreadsheet. Minitab produced the scatter plot in Figure 2.
2. The instructor fits a curve through the points. In a time-constrained situation this can be done "by eye," but can be done by hand-held statistical calculators or by computer. In each case, the students can familiarize or refamiliarize themselves with concepts of intercept, slope, and predicted values.
3. The instructor chooses two tree heights —for example, 10 m and 20 m—and determines predicted values of trunk diameter. Since the heights differ by a factor of 2, the trunk diameters should differ by a factor greater than 2 (better yet, of about 2.8) to confirm the hypothesis. In the example in Figure 2, the predicted trunk diameter values of 25 cm and 64 cm differ by a factor of 2.6, confirming the hypothesis but not reaching the value we had anticipated.

Discussion of Results

Many interesting discussion activities (and writing assignments) are possible from this project. Below are some suggestions.

1. Bring a small herbaceous plant into the classroom, preferably a dicot with a single main stem. Ask students if they have ever seen a tree that looks like that, then ask them why not. The stem of this small plant will be narrower, relative to its height, than the trunk of any large tree, relative to its height.
2. In Jonathan Swift's famous novel, Gulliver visited Lilliput, land of little people, and Brobdingnag, land of giants. Ask the students to explain in what way the Lilliputians would have looked different from ordinary humans, and why Brobdingnagians could not have existed and still look like ordinary humans. Encourage them to go beyond merely saying "they would have been too heavy."
3. In a science fiction story, a man visited another planet and saw delicate sprigs of grass 100 feet tall and 50-foot tall phalangids (daddy-longlegs) walking around on their delicate legs. Have the students explain why this scenario is physically impossible, at least on a planet with a gravitational field similar to Earth's.

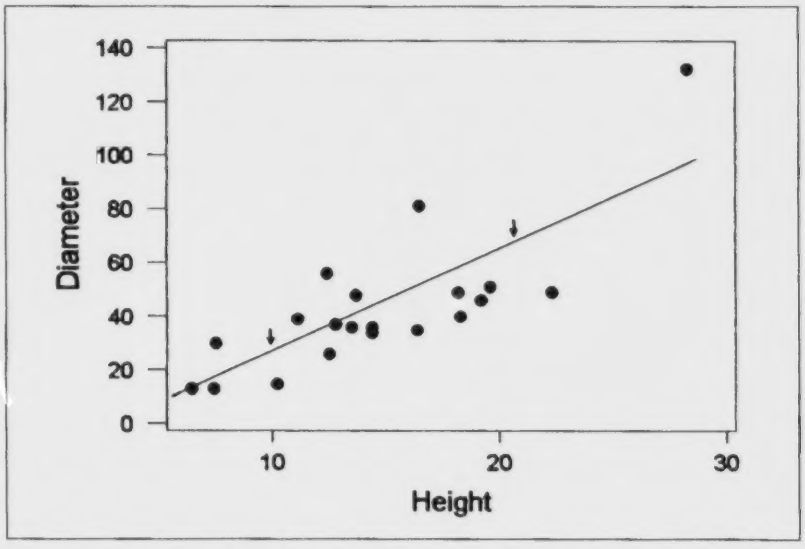

Figure 2. The relationship of tree height (m) and trunk diameter (cm) for 20 trees in a municipal park in Marshall, MN measured by botany students in 1996. Arrows indicate predicted trunk diameters for trees 10 m and 20 m in height. Minitab calculated a regression equation of Diameter = −14.0 + 38.8(height). The r^2 of 62.5% and probability of <0.001 indicated a very significant fit of the regression line.

Note that in these examples it is *not* sufficient to say that "a taller animal must have thicker legs" or "a tall tree must have a thicker trunk." This is merely a *restatement of the null hypothesis, not of the project hypothesis.*

Conclusion

The activity here described has a deceptive simplicity and is usually a pleasant outdoor activity, therefore the students enjoy it. There is, in fact, learning value in the simple enjoyment of the activity. However, after doing the calculations and discussing the results, the students find that it is not merely a pleasant afternoon outside; they discover basic biological principles that extend far beyond trees, beyond biology and beyond natural science into engineering, architecture and mathematics. They discover that all living organisms, just like buildings and cars, conform to certain mathematical principles, and that what they learn in one class may overlap and interact with what they learn in others.

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Ficus Epidermal Structures

A Tool for Introducing Leaf Anatomy

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The study of plant epidermal structures is a useful tool for introducing leaf anatomy to students. The microscopic studies (for nonscience majors) of leaf anatomy must be done with simple methods, because a complex procedure can attract the pupil's attention towards the procedure itself instead of the observation. If a microscopic procedure is complex, it may happen that the observation (which is the main target) remains relegated to a second place. Epidermal peels and freehand sectioning are simple techniques that allow one to observe stomata and the layers of the leaf without use of microtome or stains.

Both epidermal peels and freehand sectioning can be easily taught. The results these techniques offer can be useful to first-year college teachers and secondary school biology teachers.

I use easy methods in this study, understandable to everyone. The plant material used is very common (*Ficus elastica*), and it offers lots of possibilities that can be used with a pedagogical purpose. So, *Ficus* offers two interesting contrasts:

1. It has stomata in its lower epidermis, but not in its upper epidermis.
2. It shows an epidermis with several layers of cells, while in most plants the epidermis has only one layer of cells.

Materials

The materials needed are commonly available in any biology laboratory. The plant material (*Ficus elastica*) is also common since it is an indoor (outdoor in meridional regions) ornamental plant.

- Microscope slides & coverslips
- Scalpel blade #11
- Microscope
- Ordinary bleach (optional)
- Watch glass or spot plate (optional)
- Small paintbrush (optional)
- *Ficus elastica* leaves

Stomata

To prepare the study of stomata, make an epidermal peel from the underside of the leaf. Take a piece of *Ficus* leaf and try a thin cut with the scalpel blade almost horizontally; avoid cutting into the mesophyll (Figure 1). Very little green material (mesophyll) surrounded by a white epidermis means a good slice. If the cut is thick, the white epidermis area can be trimmed again. If the cut has a great amount of mesophyll, optionally, it can be bleached for 3–5 minutes on a watch glass or spot plate, washing it with water afterwards. To handle the cut, a small paintbrush is very useful. During the handling of the slice, it is possible we do not know which is the outer side and the inner side (next to the mesophyll). To avoid later troubles, it is advisable to cut the slice in two parts, turning one half upside down with the paintbrush. This must be done on the microscope slide. Then I must add just a drop of water and place the coverslip to observe.

Photo 1 shows a great amount of stomata with the guard cells clearly visible. With a bit of luck, I can obtain epidermis slices whose guard cells appear in isolation. To get effects like the ones shown in Photo 1, it is necessary to practice several cuts and observe carefully the edges of the epidermis slice. In Photo 2 a whole stoma is shown: hole and guard cells surrounded by subsidiary cells. It is possible to appreciate ordinary epidermal cells with their characteristic polygonal shape. Moreover, it is possible to observe the chloroplasts in the guard cells.

Lithocysts

To prepare the observation of lithocysts, it is necessary to make a cross

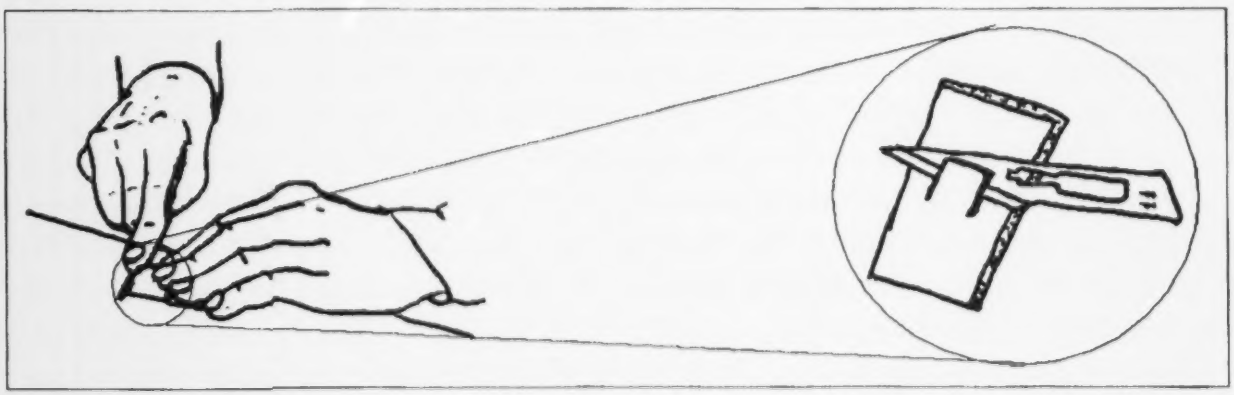

Figure 1. Taking a piece of *Ficus* epidermis from underside of the leaf.

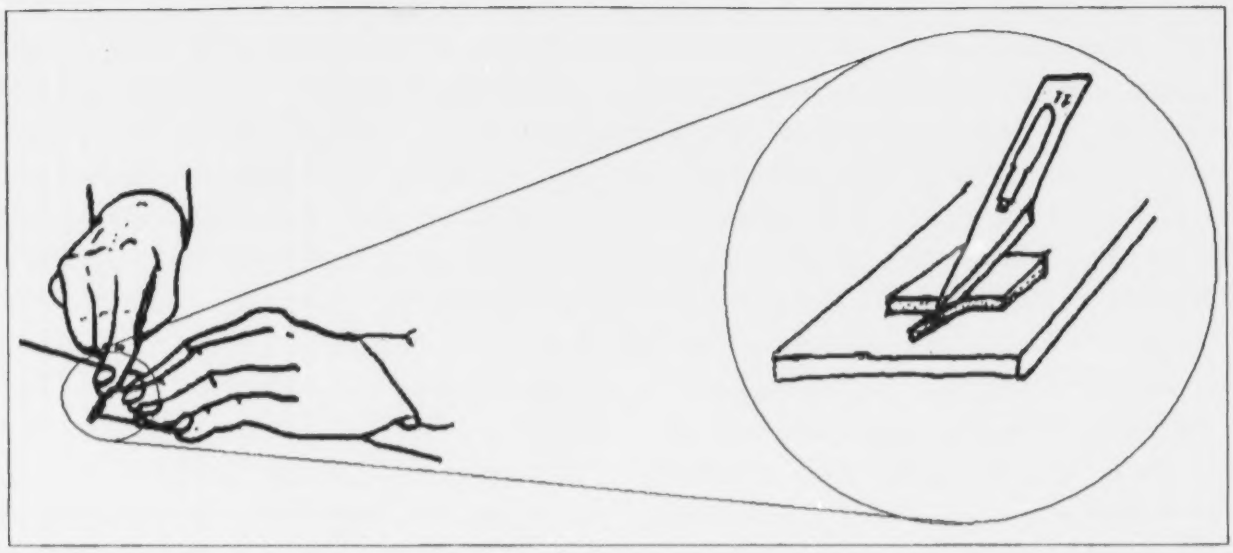

Figure 2. Making a cross cut of the *Ficus* leaf with a small piece of leaf (2 × 1 cm).

cut of the *Ficus* leaf. I take a small piece of leaf (2 × 1 cm), and I put it on the microscope slide. With the point of the scalpel blade on the glass, I will try cuts running the blade against the plant material (Figure 2). The cross cut must be as thin as possible. It is convenient to make a big cut first to even the tissue, and then to try thinner cuts. It is advisable to make several cuts to choose the best ones. Once I have selected the thinnest slices, I must add a drop of water and place the coverslip to observe.

A cross cut of a *Ficus* leaf (Photo 3) shows the thick upper epidermis in which the cuticle and several layers of epidermal cells can be seen. A big cell called lithocyst occupies all the thick upper epidermis. At magnification ×125 I can clearly see how the lithocyst goes into the palisade parenchyma. Despite the simplicity of the method, with a bit of practice, it is possible to see the foliar anatomy. Photo 3 shows the epidermis (with several layers of cells), the palisade parenchyma (which is interrupted by the presence of the lithocyst) and the spongy mesophyll. After this mesophyll, another palisade parenchyma appears, though less remarkable. Finally, I find the lower epidermis. It is an isolateral leaf.

The lithocyst is a big epidermal cell that goes into the subjacent tissues and bears a large crystal of calcium carbonate called cystolith (Photos 3 and 4). These cells are characteristic of *Moraceae, Acanthaceae* or *Cucurbitaceae* (Fahn 1985). In the *Ficus elastica* the

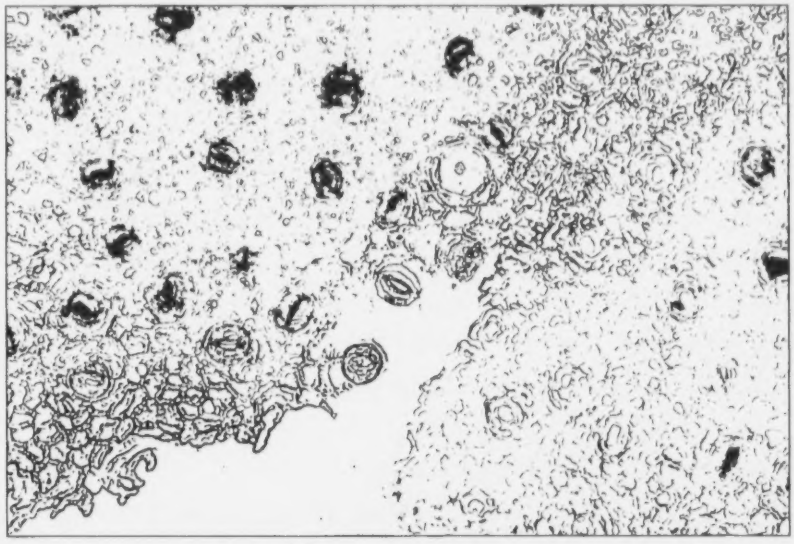

Photo 1. Lower epidermis of *Ficus elastica* (×125).

cystolith consists of a silicated pedicel from which a bunched body of cellulose and callose impregnated with calcium carbonate (>90%) hangs (Mauseth 1988). At magnification ×500 (Photo 4) the lithocyst is perfectly seen (Photo 4). Inside, a big cystolith hanging from the pedicel shows its branched structure (Photo 4).

Ficus elastica shows adaptations to water shortage, such as coriaceous leaves with a thick cuticle and an epidermis with several layers of cells. This kind of epidermis has the function of storing water, and it is probably involved in reducing water loss too (Jensen & Salisbury 1988; Raven et al. 1992). Moreover, an isolateral foliar structure is characteristic of plants growing in sunny places (Fahn 1985).

Ficus elastica, the Indian-rubber fig, occurs wild in the rain forest of India and Malaysia, where it grows into vast trees (Puserglove 1991). It would seem paradoxical that a plant which grows in wet environments presents adaptations to water shortage. But we must have in mind that *Ficus*, in wild state, usually starts life as an epiphyte and then strangles the tree on which it

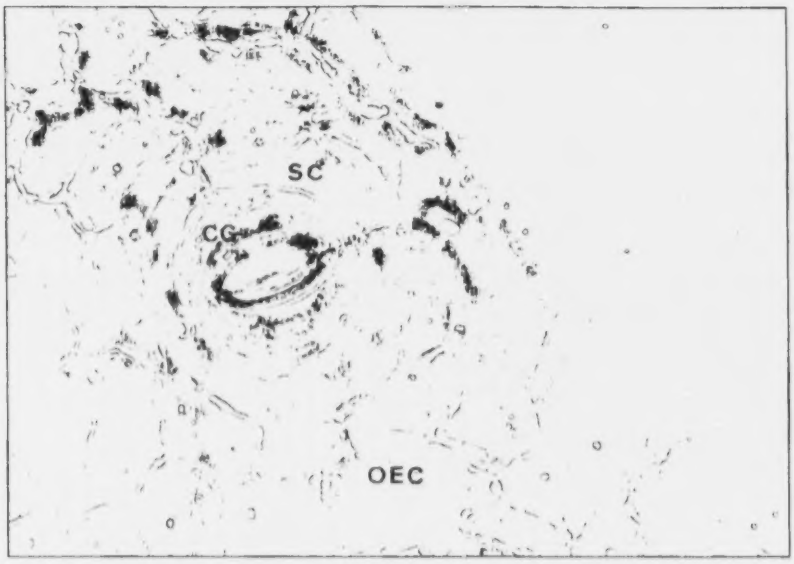

Photo 2. Stoma of *Ficus elastica* (×500). GC: Guard Cells; SC: Subsidiary Cells; OEC: Ordinary Epidermal Cells.

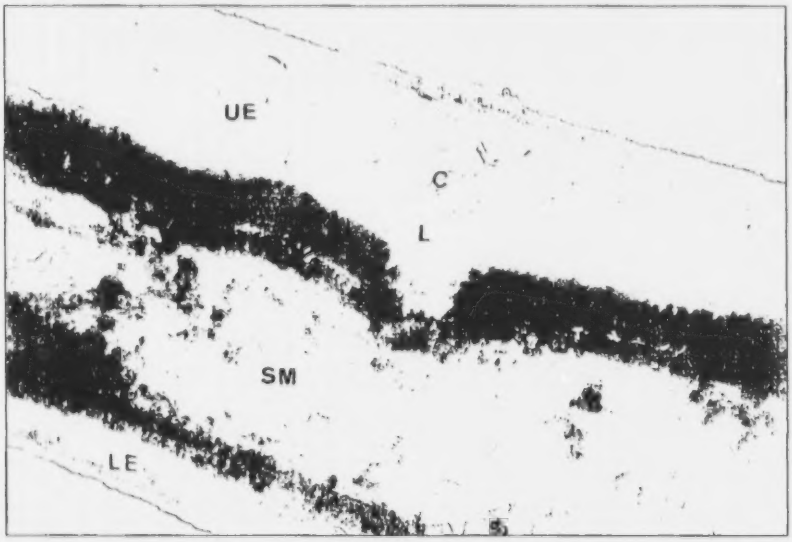

Photo 3. Leaf cross section of *Ficus elastica* (×125). UE: Upper Epidermis; PP: Palisade Parenchyma; SM: Spongy Mesophyll; LE: Lower Epidermis; L: Lithocyst; C: Cystolith.

grows. It also grows an abundance of aerial roots from the trunk (Puserglove 1991). With this way of life, the plant suffers intermittent periods of drought between storms. Moreover *Ficus* grows into a vast tree, and in this way it reaches the upper level of the rain forest, where it is sunny.

I can conclude that this paper uses easy methods that truly offer results. Moreover, I use a plant material (*Ficus elastica*) that is easily obtainable. Since *Ficus* leaves are big, and I only need a small piece, I will have enough material for a lot of students with just a leaf or two.

The method followed to observe lower epidermis stomata can be employed with the upper epidermis. In this case, *F. elastica* offers an interesting contrast, since its upper epidermis has no stomata.

Lithocysts not only appear in the *Ficus* upper epidermis, but also in the lower epidermis. In the lower epidermis there are few lithocysts, and though they are smaller, they have the same elements as those of the upper epidermis. In this way, the lithocyst also occupies the whole epidermis. It also goes into the lower palisade parenchyma and its cystolith, which though very small, shows the branched structure and its pedicel.

Photo 4. Lithocyst of *Ficus elastica* (×500). Cu: Cuticle; EC: Epidermical Cells; L: Lithocyst; C: Cystolith; P: Pedicel.

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Verifying Experiments on Photosynthesis

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Biology textbooks provide opportunities for laboratory experiments that test the facts they teach concerning various biological processes. The two primary life processes, photosynthesis and respiration, are rich with opportunities for students to test hypotheses concerning their textbook-alleged functional responses to environmental stimuli.

Respiration responses to temperature change, for example, can be easily and effectively tested on cold- and warm-blooded animals to verify if the facts attributed to these kinds of respiration are indeed true. It may thus be demonstrated, by measuring oxygen uptake, that homeotherms expend more energy than poikilotherms in keeping their internal environments "in bounds" for life; that is, that warm-bloodedness is more energetically expensive than cold-bloodedness.

The various pieces of the puzzle of photosynthesis can be subjected to a series of simple and interconnected experiments that test whether the textbook facts concerning the process are really true. Our botany class recently conducted a series of these interconnected experiments to verify our textbook's teachings about this basic process of life. Our reference texts for our series of verifying experiments on photosynthesis were the chapters concerned with photosynthesis from four texts (Raven, Evert & Eichhorn, *Biology of Plants*; Nadakavukaren & McCracken, *Botany, An Introduction to Plant Biology*; Mauseth, *Botany, An Introduction to Plant Biology*; and Stern, *Introductory Plant Biology*).

The parts of photosynthesis that our class tested were:

1. The absorbance of light by chlorophyll
2. The role of the light reaction in photosynthesis
3. Transpirational responses of C_3 and CAM plants to light and dark conditions
4. The effects of specific wavelengths of light on photosynthetic rate.

Our goals also included a discussion of the interconnections between these processes and experiments. Interconnected hypotheses related to these experiments were generated during and after the laboratory. During the lab, we informally discussed the logic of what we were doing and generated simple preliminary working hypotheses which we refined as time progressed.

Materials & Methods

Our laboratory extended over two weeks. Eleven students worked in teams of three and four to collect, and later interpret, the data. We began the experiments by first separating out chlorophylls a and b from the leaves and stems of frozen spinach. The spinach was thawed, then ground in a small quantity of acetone and boiled in a small beaker of acetone within a water bath. The resulting mixture of pigments was separated using paper chromatography with a 92.8% mixture of petroleum ether and acetone.

We cut out the chlorophyll a and b pigments from the chromatogram, eluted the pigments into 100% acetone, and determined the absorbance of chlorophyll a and b between 400 and 700 nanometers light wavelengths using a classroom spectrophotometer. Our machine was equipped with a printer so that we received an instant graph of the absorbance data. From that data, I constructed a quick and accurate hand graph so that students could see how to easily take the raw data and graph it on their own.

At the same time the chlorophylls were being analyzed, we boiled two geranium leaves in water and ethyl alcohol (within a water bath) and tested for the presence of starch using iodine. One of the leaves had been covered with aluminum foil for one week, while the other had been uncovered during that time.

We next prepared a C_3 (soybean) and a CAM (*Crassula* sp.) plant, and a control "stick-in-a-pot" for a week of light and dark transpirational water loss measurements. We tightly sealed the three pots around the lower stems of the plants with plastic bags and rubber bands, leaving the upper stems and leaves free. We weighed them on a triple-beam balance and placed them about 75 cm in front of a 150-watt lamp in a lighted room. They stayed in continuous light for 3½ days. The student lab directors were instructed to arrange for routine measurements to be taken by students throughout the week and to move the plants into a dark cabinet midway through the week. They were kept in the dark continuously, even during weight measurements, for another 3½ days.

We now prepared 12 glass petri dishes to germinate alfalfa seeds for later exposure to three regimes of 25-watt red, green and blue lights. Each group placed a fitted piece of filter paper into each petri dish, added 10 mls of distilled water, and placed five seeds into each dish; except for three control dishes that contained water and filter paper but no seeds. Seeds were germinated and grown for seven days (until thoroughly green) in white light. Each group then placed four dishes, including one control, in a one cubic-foot red, green or blue light cabinet. Light treatments were 24 hours for each color, for each group of seeds, with each of the three groups using a different order of colored light. Each group weighed each dish immediately before and after each treatment. A thermometer was also placed in each cabinet.

Hypotheses

During the initial laboratory period, alternative working hypotheses based on text information were suggested for each of the experiments. These were reinforced during the next several class periods and formal working hypotheses were agreed upon as follows:

1. Chlorophyll would absorb red and blue wavelengths of light strongly and would not absorb green light. This hypothesis was based on several graphs and paragraphs of discussion from the texts.
2. Foil-covered leaves in the light would yield a negative iodine test for starch as compared to leaves in light not covered with foil. This hypothesis was based on text discussions indicating the light reaction is a necessary prerequisite to create chemical power supplies for the dark reaction,

which in turn would synthesize carbohydrate.

3. CAM plants would transpire more water in the dark than in the light; C_3 plants would transpire more water in the light than in the dark; and C_3 plants would transpire more water in general than CAM plants. These hypotheses were based on text discussions indicating that CAM plants opened their stomates in the dark and closed them in the light, and that C_3 plants did the reverse.
4. Seedlings would gain more mass in blue light than in red light, and seedlings would lose mass in green light. These hypotheses were based on text graphs, discussions and our own laboratory spectrophotometric results, indicating that chlorophyll absorbs blue and red wavelengths of light and transmits green, and that this absorption corresponds approximately to the action spectrum of photosynthesis.

Results

Light was found to be necessary for photosynthesis and starch formation in accordance with textbook teachings. Foil-covered leaves (seven days) produced a negative iodine starch test compared to the same test given leaves not covered with foil.

Spectrophotometric data supported our hypothesis and matched textbook graphs and teachings. Spinach chlorophyll absorbed most strongly in blue wavelengths, followed by red, and exhibited essentially zero absorbance in the green.

Transpiration losses (Table 1) were about 75 times as much for our C_3 plant compared to our CAM plant. C_3 plant losses were essentially the same in the light and the dark. Dark losses for our CAM plant were about two times the losses in the light (Table 1), which was in accordance with textbook teachings.

Based on absorbance data and text discussion, we expected alfalfa seedlings to grow in accordance with observed color absorbance data. Results (Table 2 and Figure 1) partially supported our hypotheses. Red light provided a substantial weight gain in seedlings, but seedlings experienced a net weight loss in blue light. Coincidentally, temperature readings were taken in each cabinet and revealed that the red cabinet was warmest at 33.0° C, followed by green (32.8° C), then blue (31.8° C).

Table 1. Transpirational water loss measured as % of total mass loss for a C_3 and CAM potted plant in equal light and dark conditions. Plants were weighed over a seven day period.

	% Loss of Water/Day		
Plant Type	*Light*	*Dark*	*Mean*
C_3	1.93	1.63	1.78
CAM	0.017	0.030	0.024

Discussion

Spectrophotometric data and starch test observations matched textbook discussions of plant responses to light, and our hypotheses were supported. In both cases it helped us to actually observe plants in action; to see for ourselves that chlorophyll really does absorb certain colors, and then speculate on why that phenomenon has come to be (Raven et al. 1992, pp. 102–103). When we observed that plants evidently don't do photosynthesis without light, it made an immediate impression about the coupling of the sugar-making process with the light reaction. Observing the decoupling was very instructive.

Our transpiration experiments comparing CAM and C_3 plants went very much by the books. Our hypotheses and book explanations of plant responses to light and dark regimes were supported. Our C_3 plant lost 75 times as much water as our CAM plant (Table 1). Our C_3 plant lost more water in the light than in the dark; and our CAM plant, while apparently not completely closing its stomates in the light as we were led to expect, lost only one-half as much water in the light as compared to the dark.

Plants do become more and more water stressed over the duration of the experiment. Possible improvements in this experimental design include doing the dark portion of the experiment first, which minimizes initial water loss, and recharging water supplies to the plants at the beginning of each treatment.

Several aspects of this experiment are tricky. It is important to have a control pot—a stick in soil to track water losses due to evaporation of water through leaks in the plastic bags. Plant, soil and water masses should be similar for all three pots, and plant pot seals should be as tight as possible. Potted plants should be left unwatered for several days prior to the start of the experiment, and at the start, known amounts of water (50 mls) should be added to each pot.

Some additional ideas for experiments are to compare C_3 plants (soybean) to C_4 (corn). It is instructive to use plants that have photosynthetic regimes which are not known to the experimenters. We found to our surprise, for example, that magnolia (*Magnolia grandiflora*) acts like a CAM plant. More (student) experimentation might need to be done with this plant (and others).

The growth of alfalfa seedlings in colored lights (Table 2 and Figure 1) was at variance with our previous data, working hypotheses, and textbook discussions of plant responses to color. Both the texts and our spectrographic data indicated that plants would grow very little, or not at all, under green light. We found no net growth, on average, under either green or blue light. Our hypothesis based on text teaching guessed that blue light would provide the greatest growth of the three light colors. Possible explanations of the results include: error in measurement, variable wavelengths within the light emanating from the bulb, and spilled water from petri dishes. Or it may be that individual plants are variable in their use of color. We found positive *and* negative growth rates under all three colors (Table 2). We varied the order of the light treatments for each of the groups. So, it may be that plants respond differently to light treatments depending on the wavelengths they "see" first. A variation to our experimental procedure is to allow each set of seeds and each control to experience only one color of light.

Table 2. Mean (n = 3) % weight change of seedlings grown in nine petri dishes under red, green and blue light wavelengths for 24 hours per color. Each petri dish contained five seeds.

Group	*I*	*II*	*III*	*Mean*
Lt. Color		Percent Weight Change		
Red	+0.31	−0.18	+0.22	+0.12
Green	−0.32	+0.11	+0.06	−0.05
Blue	+0.20	−0.08	−0.18	−0.02
Mean	+0.06	−0.05	+0.03	+0.015

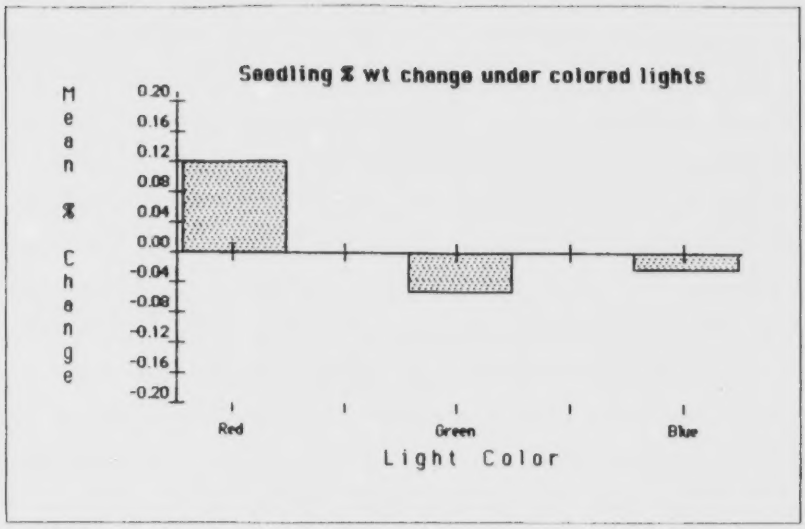

Figure 1. Mean (n = 3) % weight change of seedlings grown in nine petri dishes under red, green and blue light wavelengths for 24 hours per color. Each petri dish contained five seeds.

Statistical tests could be calculated to check for seedling variability between petri dishes, and it would be instructive to students; but it is also simply instructive to note the variations from textbook teachings and then speculate why this is so. We concluded that textbooks may generalize too much on plant responses to light wavelengths.

Temperature differences within each cabinet may also have affected our results by affecting the rate of respiration of the seedlings under each light. Red was the warmest, followed by green, then blue. The temperature differences may also have affected the dark reaction of photosynthesis under each light color. These temperature differences may cause offsetting effects in photosynthesis and respiration. Students may then cogitate on possible experiments that could be done to address these questions/problems.

Conclusion

Explanations other than those given to explain all of our results are, of course, possible. I tried to provide students with as many alternative explanations as I could think of to explain divergences of actual results from results to be expected from text descriptions. This approach, relating lab results to text explanations, teaches students that there are no pat yes and no answers to the "whys" and "hows" of biological phenomena.

It is a challenge both to teach and to write this lab, and it is especially challenging to write it in an interconnected way. Students must be encouraged and monitored at all steps and stages along the way. I suggested to students that they write in a way that they feel most comfortable with. They may choose to write each part separately or they may write it as a group of related experiments. Either way I constantly stressed the importance of noting and describing interconnections of various parts of the lab to each other, and I required them to use standard scientific format in their write-ups.

With this hands-on experience in experimental verification of textbook-explained processes, students begin to feel connected to the process of scientific discovery rather than simply passive recipients of previously discovered knowledge. They also learn about the amount of hard work and rigor that experimental science involves; and they become better critical thinkers of science in particular and academic subjects in general.

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Samara Dispersal in Boxelder

An Exercise in Hypothesis Testing

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Pedagogical surveys have shown that traditional approaches to teaching botany, with their emphasis on preserved specimens, prepared slides and slow-to-develop physiological experiments, tend to lead students to one conclusion—plants are boring (Uno 1994). Surveys have also shown that students have a strong preference for hands-on experiments which utilize living material and generate fast results. The incorporation of such experiments into botany or ecology exercises is especially difficult in the "dead" of winter in northern climes. We present here a fun, inexpensive and pedagogically useful laboratory exercise which involves indoor studies of the dispersal properties of the winged fruits (samaras) of boxelder (*Acer negundo* L.) trees. The exercise engages students in the processes of hypothesis testing, experimental design, and data analysis, as well as introducing them to some important concepts relating to functional ecology, succession and plant reproductive biology.

An attractive feature of the exercise presented is its flexibility. If scheduling and the abilities of the students permit, the exercise can easily be re-tailored to incorporate more independent inquiry on the part of the students; alternatively, it could be pared down to be a highly-regimented "cookbook-type" lab. We have adapted sort of a middle ground between these two approaches, so that the exercise does require independent inquiry by the students, but can also be completed in a single, three-hour lab period. In our particular case, a third of this time is devoted to a discussion of statistical analysis (*t*-tests), a step perhaps not necessary at the pre-college level and not further elaborated upon here. For those instructors who can devote more than one lab period to this exercise and wish to use it as the basis for independent projects by their students, we refer readers to an earlier How-To-Do-It publication in this journal (Thomson & Neal 1989) which presents additional pedagogical exercises pertaining to the wind dispersal of fruits and seeds, including the construction of artificial paper samaras, studies of the effects of partial dissections on the dispersal properties of tree-of-heaven (*Ailanthus*) samaras, and the collection in transects of spray-painted samaras dropped from a flagpole. The practical advantage of the present exercise is that it can be completed indoors in a single lab period in a typical classroom (no flagpoles, catwalks or balconies necessary).

Boxelder Samaras

Samaras are winged fruits morphologically adapted for wind dispersal. The samaras of many species, including ashes (*Fraxinus* sp.), elms (*Ulmus* sp.) and maples (*Acer* sp.) spontaneously develop a spinning motion soon after being dropped. The functional significance of this "autorotation" is to produce lift and drag to counter the forces of gravity, thereby reducing the falling speed of the fruit and increasing the distance it may be transported by horizontal winds (Augspurger 1986). The longer a samara is airborne, the greater are its chances for settling in a gap in the forest canopy far from the shading limbs of the source plant. The selection pressure behind autorotation is thus directed toward improvement of the dispersal potential of the plant.

Boxelder is a dioecious, wind-pollinated, arborescent member of the maple family (Aceraceae), which has a wide distribution in the temperate regions of non-coastal North America. Although other samara-bearing species might conceivably be adapted for use in this exercise, boxelder has four advantages which might not be met by the samaras of other species:

1. Boxelder has a prolonged fruit dispersal season, typically from mid-autumn to early spring, which enables the samaras to be readily located and collected in undamaged form during the "dead" of winter.
2. The samaras are dry at maturity and may descend less quickly than the fleshy samaras of other species. This permits their descent to be measured with a stopwatch in a typical classroom.
3. Unlike most plants, boxelders retain their sterile fruits rather than aborting them, and these sterile samaras develop in parallel with the fertile samaras. Because of insect damage, spontaneous abortion and the relative inefficiency of wind pollination, a large and variable percentage (20 to 60% in our experience) of boxelder samaras are sterile. Thus, the dispersal properties of two naturally occurring samara subpopulations can be studied.
4. Boxelder samaras are of equal area regardless of whether they are sterile or fertile. Sterility in boxelder fruit is cryptic: it is not easy to distinguish between fertile and sterile samaras without a destructive dissection of the seed chamber. Since visible inspection cannot be used reliably to distinguish between fertile and sterile samaras, the dispersal experiments are essentially done "blind."

Samara collection may be done by the teacher alone or may be incorporated into an earlier exercise involving a nature walk, tree-keying exercise, or other outdoor activity. Boxelders are commonly found growing along riverbanks, or along the borders of moist fields or railroad embankments. Boxelder often serves as an invading pioneer species during the succession of old fields. During forest succession, boxelders usually succumb prior to the oak-hickory climax stage, probably as a result of too much shading. The samaras of boxelder are paired and about 2.5 to 3.5 cm in length, and occur on drooping racemes 15 to 25 cm long. The natural history of boxelder is well-summarized by Maeglin and Ohmann

(1973). It is advisable to collect the samaras a few days before use and let them dry uniformly, especially in the early autumn when the moisture content of the naturally drying samaras is quite variable: fresh, green, fleshy samaras are not suitable. Also, because there can be considerable intraspecific variation in the morphological attributes of *Acer* samaras (Sipe & Linnerooth 1995), it is advisable to collect and pool the samaras from several different specimens.

Three Hypotheses

In this exercise, the students are asked to consider three different hypotheses concerning the respective dispersal of sterile and fertile boxelder samaras.

Hypothesis I is based on the assumption that it would be advantageous to have sterile fruits fall closer to the source plant. Because sterile fruits have zero reproductive potential, they are essentially a lost investment for the source plant. Some of this lost investment might conceivably be recouped if the sterile fruits contributed to the "leaf-litter" immediately beneath the source plants. Indeed, Otto and Nilsson (1981), in attempting to explain the winter-time retention of lower canopy leaves in beeches and oaks, proposed that the delayed shedding of the previous year's leaves may benefit the tree by providing the nearby ground, well-leached by winter's thaw, a fresh supply of soluble nutrients in the early spring. *Hypothesis I, therefore, predicts that the optimal reproductive strategy for species such as boxelder, would be to have fertile samaras land farther from the parental plants and to have sterile samaras fall closer.*

Hypothesis II is based on the notion that ability of boxelder seeds to colonize an old field is limited chiefly by post-dispersal seed predation. Studies of old field colonization have shown that the largest mortality of colonizing tree species is due to predation of seeds by rodents (Gill & Marks 1991). Conceivably, the prolonged dispersal of boxelder fruits over the course of winter may be an evolutionary adaptation for reducing seed predation by winter-foraging mice. The "cryptic sterility" of boxelder samaras may also lower their desirability as a primary food source and may act, therefore, as an effective adaptation for lowering post-dispersal seed predation. If so, then this strategy of "cryptic sterility" would be most effective if the post-dispersal mixing of sterile and fertile samaras were maximal. *Hypothesis II, therefore, predicts that sterile seeds should fall the same distance from the parent as do fertile samaras.*

Hypothesis III is based on the findings of researchers such as Green (1980) who have found that the rate of descent of samaras is highly correlated with the square root of the samara's wing-loading (samara mass divided by wing-surface area). For example, Augspurger and Hogan (1983) found that fruits of *Lonchocarpus* with 0, 1, 2 or 3 seeds had increasingly large values both for $(\text{wing-loading})^{1/2}$ and for rate of descent in still air. Therefore, based on the reasonable assumption that fertile samaras weigh more than sterile ones, *Hypothesis III predicts that fertile samaras, bearing more wing load, should fall closer to the parent than do sterile samaras.*

After having the three hypotheses explained to them, the students are asked to break into groups of three and devise an experiment to decide between them. The students are also encouraged to make preliminary observations concerning the determination of the reproductive status of the samaras, as well as the fruits' general autorotary properties. It should be pointed out to them that samara descent is a two-step process consisting of a rapid, initial "free fall" of about 20 cm, followed by a much slower autorotating descent to the ground.

Experimental Design

Based on their training and inclinations, the students propose a multitude of diverse approaches, some more feasible and some more imaginative than others, to test the predictions of the three hypotheses.

One of the more common suggestions involves collecting the fallen fruits in transects surrounding specimens of boxelders, noting the distances between the site of collection and the presumed parent plants, and then determining the reproductive status of the fruit. Although this research strategy has the advantage of being most natural, questions arise over the uncertainty of assuming that the closest specimen is indeed the source of all the collected fruits and, whether the presumably more nutritious fertile fruits might not be subject to enhanced predation. Although both of these problems are surmountable, their resolution might require many months of outdoor study. It is more feasible to study the question indoors using collected fruits.

Another common proposal involves measuring the differences in the dispersal properties of fertile samaras before and after the seeds have been surgically removed. This approach, too, is discouraged because it is no trivial matter to remove the seed without damaging the samara, and, moreover, such manipulations might well alter the samaras' aerodynamic properties.

Students must also confront the problem of what parameter to measure. Many students propose measuring the horizontal distance a dropped samara travels from the plumbline. However, because of the vagaries and weakness of indoor air currents, this approach, too, is discouraged: the samaras when dropped in still air generally fall close to the plumbline. The simplest and most straightforward suggestion is to measure the time needed for the descent of dropped samaras in still air, and then to determine whether they are sterile or fertile.

A further complication arises because the descent of autorotating samaras has two stages, a rapid free fall and a slower terminal velocity. Because the terminal velocity of the samara is the best descriptor of autorotary descent, and because the very rapid free fall makes little contribution to the total time needed to descend, some authors (e.g. Thomson & Neal 1989) have recommended estimating the rate of descent by dividing height by time. The appropriateness of this simplifying assumption is the topic of one of the questions for discussion. Because there are two velocities involved in descent, however, we prefer to have our students record and present their data as time required to descend a given distance (2.4 m or 8 ft in our case).

Students are also confronted with problems associated with the statistical analysis of their data. One of the more common sources of error in data collection and analysis arises from the non-independence of data values (i.e. pseudoreplication). Students are often tempted to treat repeated measurements taken from the samara as independent values during statistical analysis. This error arises from the misconception that the aim of scientific observation or experiment is to obtain a large number of measurements rather than measurements from a large number of subjects. True replication of measurements, however, is necessary to guard against aberrant single values. We recommend that each group of students measure the descent time of 30 different samaras three times each and

determine the average descent time for each of the 30 samaras.

We have found that students can get useful results by standing atop laboratory benches (much to their delight) and dropping samaras from a position parallel and adjacent to the ceiling. (For safety concerns, the samara droppers should wear suitable shoes while standing atop the table). A second student measures the time of descent with a stopwatch (0.01 second resolution), while a third records the data. To avoid mistakes and confusion, it is a good idea to have the students number their samaras with a fine-tipped, indelible marker prior to experimentation. Students should be reminded that each samara should be dropped and timed in a uniform manner to reduce experimental error. The exercise can also be expedited by supplying the students with a standard data sheet. The difference between the descent times of fertile and sterile samaras can be visualized graphically or, in advanced classes, analyzed statistically by means of a two-sample *t*-test (Table 1) as explained in any introductory statistics textbook (see e.g. Sokal & Rohlff 1995).

Another salient property that can be measured is samara mass (Table 1). Although usable measurements can be achieved with a typical laboratory pan balance with a resolution of 0.01 gm, we recommend using an analytical balance. Not too surprisingly, fertile samaras weigh more on average than do sterile samaras (Table 1). Plotting samara mass v. time needed to descend reveals a clear inverse relationship ($r = -0.66$) between the two: fertile samaras fall faster (see Figure 1.) The predictions of Hypotheses I and II are rejected, and the prediction of Hypothesis III is supported.

To calculate wing-loading, the surface area of the samaras must be determined. This parameter can be estimated by photocopying the samaras, and comparing the mass of the samaras' individual photocopied images to the mass and area of the photocopied sheet. An analytical balance (0.0001 g resolution) must be used to measure the weight of the photocopied samaras. Cutting out the samara images and weighing them is tedious and also doubles the length of the exercise. Because of time constraints, it may be preferable to inform the students that previous researchers (this study) have determined no significant difference between the surface area of sterile and fertile boxelder samaras (see Table 1).

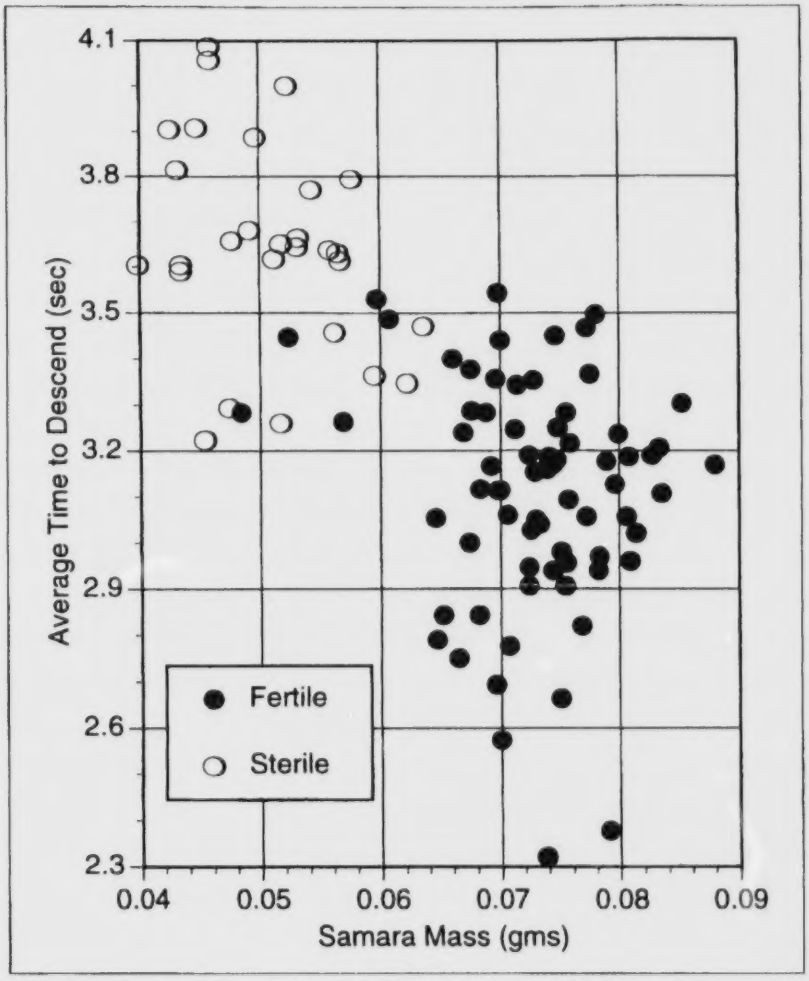

Figure 1. Relationship between boxelder samara mass and time needed to descend 2.4 m (n = 100). Note the clear separation between the sub-populations of fertile and sterile samaras.

Table 1. Summary of experimental results demonstrating that fertile boxelder samaras on average weighed more and descended a distance of 2.4 m more quickly than did sterile samaras. No significant difference was found between the average surface areas of fertile and sterile samaras.

	Number (n)	Average Mass (gm) ± Standard Deviation	Average Surface Area (cm^2) ± *Standard Deviation*	*Average Descent Time (sec)* ± *Standard Deviation*
FERTILE	72	0.0727 ± 0.0070	3.27 ± 0.35	3.11 ± 0.25
STERILE	28	0.0508 ± 0.0062	3.24 ± 0.42	3.65 ± 0.23
TWO SAMPLE *T*-TEST		$t = 15.28$ significant; $P < 0.001$	$t = 0.89$ not significant; $P > 0.05$	$t = 19.29$ significant; $P < 0.001$

When the (wing-loading)$^{1/2}$ parameters for the data points presented in Figure 1 were plotted vs. time needed to descend, an even stronger correlation ($r = -0.75$) was determined (data not shown).

The experiment's support of Hypothesis III offers two important lessons. The first is that hypotheses, no matter how intuitively attractive they are, need to be objectively tested. The second is that there are limits to evolution. Even though boxelders might benefit if the dispersal strategies suggested by Hypotheses I or II were correct, this does not mean that boxelders have evolved or will evolve these strategies. There are morphological, developmental and physical constraints to evolution. Because of such constraints, birds have not evolved helium-filled sacs and cats have not evolved stainless steel claws. Similarly, because of aerodynamic constraints, boxelders have not evolved sterile fruits that fall closer to the source plant.

Questions for Discussion

1. In our experimental design, we ignored the fact that there are two components to the descent of samaras: a very rapid free fall and a slower autorotation (the terminal velocity). Propose an experiment to determine whether the differences between the descent of sterile and fertile samaras are due to differences in the length of the free fall or in the terminal velocity.
2. Guries and Nordheim (1984) have suggested that blade length is an important parameter in determining the length of a samara's free fall. Samaras with long blades require a longer initial free fall than do samaras with short blades. In what ways would you expect the samaras of a small maple species such as *Acer pennsylvanicum* (striped maple or moosewood) to differ from those of a tall species such as sugar maple (*Acer saccharum*) or red maple (*Acer rubrum*)?
3. Like many wind-pollinated trees in the temperate zone, boxelder forms flowers in the early spring before the leaves expand. What is a possible advantage of releasing pollen before the leaves expand? With this in mind, how might boxelders benefit aerodynamically by releasing their samaras during winter?
4. Can you think of any other common plants that use wind to disperse their seed or fruit? Do these seeds or fruits also autorotate?
5. If lighter seed masses cause slower rates of samara descent and greater seed dispersal, would individual boxelders that produce on average smaller seeds than normal be at a selective advantage? Why or why not?

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Make Some Porometers & Use Them To Determine When Your Plants Are "Working"

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Perhaps one of the reasons that plants get slighted, both in and out of school, is that they seem to just sit there. Things can be seen to happen over days or weeks, but over the span of a lab exercise or a short look they don't seem to be doing anything at all.

Of course a healthy leaf in sunlight is actually working at a great rate: It's taking up CO_2 from the air and making sugars and starch. And since that leaf has to present a wet cell surface to the air in order to have the gaseous CO_2 dissolve in it, it must be losing water to the air at the same time. These coupled processes of photosynthesis and transpiration couldn't be more important. They're also very dynamic phenomena that are constantly responding to light, soil, water, temperature, humidity, etc. The problem is that all these interesting relationships are usually invisible to us. The problem is to be able to measure at least relative rates of photosynthesis and transpiration—in a sense, to see them happen and make the plants come alive.

The solution depends on the fact that those critical fluxes of CO_2 and water vapor are very largely under the control of the plant. Almost the only way that gases can get in or out of a leaf is through a large number of valves on the leaf surface called stomata. When those valves are closed, the fluxes of CO_2 and water vapor are effectively stopped. For most plants this means photosynthesis is stopped as well—they really are just sitting there. When stomata are open most plants are photosynthesizing and necessarily losing water through transpiration as well.

It follows that being able to monitor the degree of stomatal opening is an extremely important measurement. You can do this by simply looking at stomatal apertures under a microscope, but this technique is usually destructive, time consuming, and difficult to quantify. A better method is to use a porometer to measure how "porous" the leaf is. The most sophisticated types measure the rate at which water vapor diffuses out of a leaf into a chamber of known humidity. However, these are very complex instruments and they have their own problems, one of which is cost. A much simpler type of porometer measures how rapidly a certain quantity of air can be driven through a leaf by a known pressure difference. These air flow porometers are often referred to as "viscous flow" or "mass flow" porometers since they measure not diffusion but a bulk or mass flow of air.

The air flow porometer I'll describe is cheap and relatively simple to build. A measurement generally takes only a few seconds to a minute, is nondestructive, and yields a resistance to air flow which is a very sensitive indicator of the relative degree of stomatal opening. Its primary limitation is that it's restricted to leaves which have relatively smooth surfaces and with stomata on both top and bottom. This unfortunately rules out most trees and shrubs, since these have stomata on the lower surface of their leaves only, but it does allow measurements on the majority of herbaceous species. Corn and sunflower work especially well, and so will many common wild plants found outdoors, including clovers, grasses, dandelions and goldenrods. Simply looking at any one of these plants tells us nothing about whether they're "working." As simple as the measurement of resistance to air flow across a leaf is, it yields very important information. Moreover, it can open up to students and teachers a fascinating and intricate control system that we still know very little about. How stomata respond to light, CO_2, water status, air pollutants, etc. are real and current problems in biology; they are not lab exercises and therefore are inherently more interesting.

Air Flow Porometers

A simple air flow porometer that used a falling water column to draw air out of a leaf was used by Darwin's son Francis over 80 years ago (Darwin & Pertz 1911). Their results, which showed how the resistance to air flow varied with light and water status, are still interesting. The main problem with this type of instrument is that a chamber has to be glued onto the leaf surface in order to ensure an adequate seal. This prevents sampling a number of leaves and discourages working outdoors. More importantly, there is no easy way of ensuring that the permanent chamber attached to the leaf isn't influencing the stomata that you're trying to measure. It's a great advantage to have an instrument that can make rapid measurements and be off each leaf before the stomata have time to respond.

Many designs since then have used rubber seals that can be clamped on the leaf; these are well reviewed by Meidner (1981). Some are not instruments that could be easily built or used in school or in the field. However, Meidner (1965) developed a simple model for school use that pulled air through the leaf by an expanding rubber bulb. Alvim (1965) developed a rugged instrument for field use designed around a sphygmomanometer bulb and gauge. Milburn (1979) published a very compact and simple design in which the driving force for air is a high vacuum created by withdrawing a syringe.

The main difficulty in working with instruments that quickly clamp and unclamp from a leaf is the practical one of routinely establishing a dependable seal at the leaf surface without damaging the leaf. This is the absolute prerequisite for knowing that the very small air flow we're measuring is strictly through the leaf. It does little good to test an instrument on some impermeable plastic or metal, because what you have to know is that you've established a seal on the actual leaf that you're making a measurement on. If the leaf has prominent veins or hairs or the air pressure is too high relative to the clamping pressure, then the leaf to gasket seal may leak.

In the instrument I'll describe, the pressure difference that drives the air is very small—equivalent to a water column about 8-cm high. This minimizes the clamping pressure required

to prevent leaks. Further, the leaf to gasket seal is always tested before a measurement in the following way: assume that the air forced in one side of the leaf all travels directly across and exits the opposite surface. When chambers are tightly sealed on opposite sides of this leaf and pressure is applied to one chamber, but prevented from exiting the other, there should be no air movement. This, in fact, is the arrangement before every measurement. Only after the seals are checked in this way is the exit chamber opened and the measurement begun.

What about the assumption that all the air goes directly across the leaf? With thick leaves air may also move laterally through the leaf tissue (Meldner 1981). However, with thin leaves and low pressure gradients this lateral movement must normally be negligible. The instrument I'll describe, when used with many non-succulent leaves that have stomata on both surfaces shows no air movement when the exit chamber is blocked. Further, when it's used to apply pressure to the lower leaf surface of a species which has stomata on the bottom only, non-succulent leaves never show any air movement. Thicker leaves do, but there is no way of routinely checking (in this case) that the leaf to gasket seal is absolute. So, again, the instrument is effectively limited to species that have stomata on both sides of the leaf.

Building a Simple Instrument

To make the simple porometer shown in Figure 1 you'll need two small pieces of lucite glued to a hinge. The lower piece has a hole drilled in it into which a 15-cm long capillary is glued. (A 1-ml pipet works well.) The lower end of the capillary goes through an oversized hole in a cork. The cork goes into a clear plastic test tube which is 3/4 filled with water. The water chamber is always at ambient pressure because of the oversized hole in the cork. The air pressure in the capillary will be adjusted by sliding the test tube and cork up and down the capillary, so the latter should also have a piece of rubber tubing on the bottom as a stop. The upper lucite also has a hole drilled in it with a small flap of rubber over it. This is where the air exits after traveling across the leaf. It is important to be able to block the exit tightly to verify that the leaf to gasket seal doesn't leak. The air fluxes involved are so small that the rubber is required for a dependable seal.

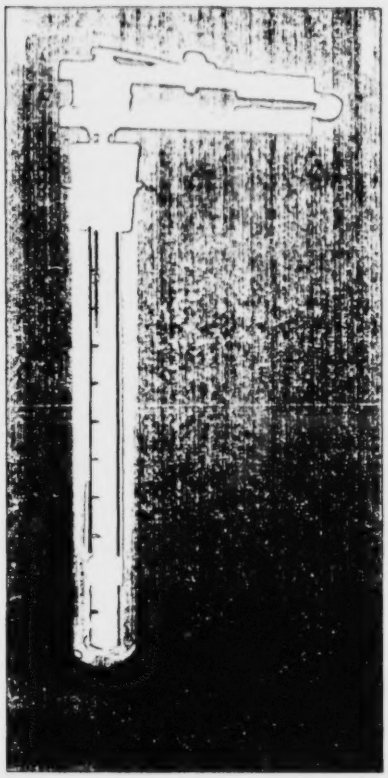

Figure 1. The simplest porometer has a test tube for a water chamber. A capillary attached to the lower clamp surface is immersed in the water chamber and can be emptied by sliding the test tube down. When the clamp is fixed on a leaf the test tube is slid back up and water moves up the capillary only as air is forced through the leaf.

Between the lucite pieces are two matching soft rubber washers. These gaskets are really at the heart of the problem of getting a good seal without damaging the leaf. I've used a clear silicone rubber compound (GE Silicone II) which can be bought anywhere and is generally soft enough. It requires air to set up so the easiest way to make some gaskets is to put some mounds of silicone on a glass sheet and let them fully cure a few days. Then pull off the silicone carefully; the lower smooth surface will contact the leaf. The opposite surface can be trimmed to establish the thickness (whatever is required to make the gaskets parallel when the clamp is closed), and the inner and outer diameters cut out with cork borers. For the instrument pictured in Figure 1, each gasket is 3-mm thick and has an inner diameter of 3 mm and an outer diameter of 12 mm. Smaller gaskets can be advantageous for getting between the major veins of some leaves. Narrow ones work well for grasses.

The instrument described above is the simplest to build and use; but if you need to measure leaves that are very close to the ground, the test tube gets in the way. A version with a remote leaf clamp is shown in Figure 2. Here the capillary is surrounded by a piece of tygon tubing which acts as the water reservoir. The plastic tee at the top over which the tygon fits has an air channel filed into it to keep the water chamber at ambient pressure. The capillary is evacuated prior to measurement by moving the syringe down. This system has the advantage of being able to evacuate finer capillaries than gravity alone does and hence allows a more sensitive instrument if it's required. The capillary in the instrument shown in Figure 2 has an internal volume of approximately .02 cm^3 per cm of length, so volume changes as small as .002 cm^3 can be distinguished.

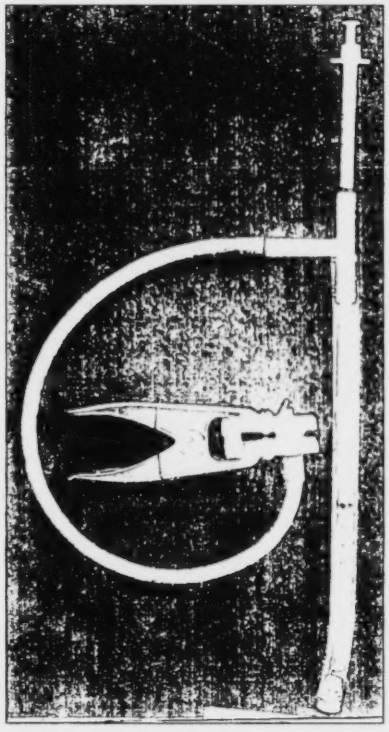

Figure 2. A slightly different design which separates the leaf clamp and manometer. The latter is, again, a capillary within a water-filled tube (here tygon) but the water is forced out of the capillary prior to measurement by a syringe.

Another modification shown in Figure 2 is a spring clamp which generates a constant gasket pressure. This might have advantages in some cases but when working with a variety of leaf types it is actually more convenient to be able to vary the clamp pressure by hand.

Using the Porometer

To use the instrument shown in Figure 1 the first step is to slide the water chamber (test tube) down until it contacts the rubber stop on the bottom of the capillary. This empties the capillary. (If the capillary is glass and has a narrow bore, add a tiny amount of liquid soap in the water to keep the water from hanging up.) Then close the hinged clamp somewhere on the leaf surface that avoids major veins and block the exit hole at the top with one finger. Next, generate the driving pressure in the trapped air within the capillary below either by sliding the water chamber up the capillary (Figure 1) or by pushing down on the syringe (Figure 2). If you see water rise back up the capillary, air is escaping through the leaf to gasket seal—try applying more pressure on the clamp. When the meniscus is fully stationary then you have achieved a seal and are ready to start the measurement. Remove your finger from the upper exit hole and the water column will rise in the capillary as air is driven through the leaf. Time the rise of the meniscus over some increment in the capillary with a watch or simply count.

Though not as simple as the technique above, you can actually tolerate some air leakage (meniscus movement with your finger blocking the exit) if it is at a constant rate and is much smaller than the rate with the exit chamber opened. Subtract first the air leakage rate from the second to estimate the air traveling across the leaf. However, even very high clamp pressures may not achieve a workable seal on leaves with protruding veins or stiff hairs. If you really have to work with such a species, try putting a little water on the gasket surface before each measurement. If that doesn't work you can use Vaseline.™

It's actually a good idea to have students start using the instrument by measuring the porosity of paper instead of leaves. Without the complication of real leaf surfaces and varying stomatal apertures, students can become familiar with the use of the instrument and get a feel for the physics and the very small volumes of air flow involved. They'll be surprised at how sensitive a flow meter this instrument is. Most newsprint and nonglossy papers will be shown to be very porous; they generally have a resistance to air flow lower than any leaf you'll be working with.

Resistance to air flow can be expressed most simply as seconds required for the meniscus to move a fixed amount. This works fine as a relative measure as long as you're working with only one porometer design. If, instead, you need to account for differences in driving pressure, leaf area, or volume of air moved, then you'll have to standardize the results in units of resistance as described in the Appendix.

Investigating Stomatal Response

When starting to work with plants make sure that you have leaves that allow a complete seal and have stomata on both sides. Experiment with different species. After finding some that work well you could begin by investigating how resistance varies with non-environmental variables:

- Does the resistance depend on leaf age? Very young or old leaves generally show higher resistance.
- Does the resistance vary over different parts of the leaf? Sections of large leaves that are more remote from the source of water might be at a lower water status.
- Do repeated measurements on the same leaf or leaf sections influence the results?

Measurements repeated frequently enough at one spot are likely to cause the resistance to go up, probably due to shading. Once you get a feel for these effects you can define a set of leaves and sampling locations that can be used for investigating the response to environmental variables. There are good reviews of these in Jarvis and Mansfield (1981), Wilmer (1983), and Zeiger et al. (1987).

The first response that comes to mind is probably stomatal closure due to plant water stress. This is what stomata were designed to protect against. To demonstrate the instrument shown in Figure 1 the resistances of upper leaves of a sunflower in full sun were measured before and after cutting the stem. The stomata started to close about 5 minutes later (Figure 3). Note that this was considerably after the leaves had become completely wilted. Leaves drying at a slower, more natural rate usually start to close their stomata before severe wilting and this in fact may operate through a different mechanism. It's been suggested that stomata are typically responding not to the water status of the bulk of the leaf at all but rather, in a sense, are anticipating water problems for the leaf by responding to cues at opposite and extreme ends of the water pathway: The water status of epidermal cells that are in direct contact with the drying air and the water status of the soil (actually roots?) which is communicated to the leaves via a chemical messenger. This is an interesting idea with a lot of implications. You can test it.

Light is another fundamental variable. When there isn't enough light for photosynthesis there is no need to lose water, so stomata normally close. Hence the stomata of most species close at night. Figure 4 shows the effect of moving potted sunflower plants from full sun to the extremely low light under a counter in a classroom. Within 10 minutes the leaves had significantly higher resistance. Note, though, that they were far from completely closed, even after 30 minutes. This is a typical result which is often explained by an underlying endogenous rhythm. Plants aren't so easily fooled: Low light at the end of the day will initiate complete closure, low light in the middle of the day won't.

Upon returning the plants to full sun the stomata reopened within 15 minutes but before that they temporarily closed (Figure 4), probably due to a transient water deficit caused by the step change in transpiration.

Stomata also may close in response to high ambient CO_2 (watch out for too many students in a small plant room), low humidity, high and low temperature, disease, and nutrient deficiency. Some species adapted to dry conditions open their stomata only at night. Any of these are worth looking into. You can try adding chemicals to the water supply or leaf surface or measure the effectiveness of physical antitranspirants (e.g. "wilt-pruf") that cover the stomatal pores with wax. You can see whether resistance varies with plant variety or with growing conditions, e.g. growing in wet vs. dry environments or in the field vs. a windowsill. When there are such differences do they correlate with differences in stomatal density as determined with a microscope?

There's no end of interesting investigations that can be done here. Stomata are part of a fascinating control system that becomes more dynamic and interesting the more we know about it. Understanding that system isn't that simple, but that's what makes it interesting.

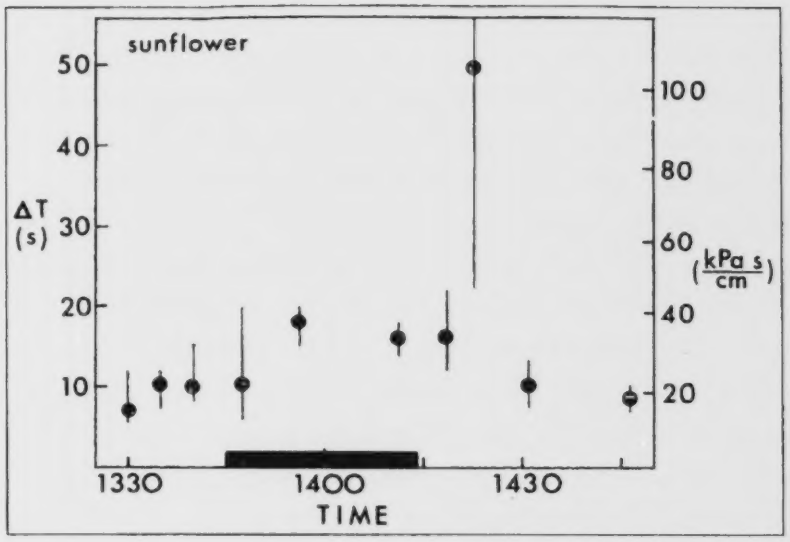

Figure 3. The response of the upper leaves of a potted sunflower when the shoot was cut and left to dry in the sun. The left axis shows the time required for the porometer's meniscus to move a fixed amount, and this corresponds to a fixed volume of air (here 0.12 cm^3) forced across the leaf. The right axis expresses the result in units of resistance, R, as described in the Appendix.

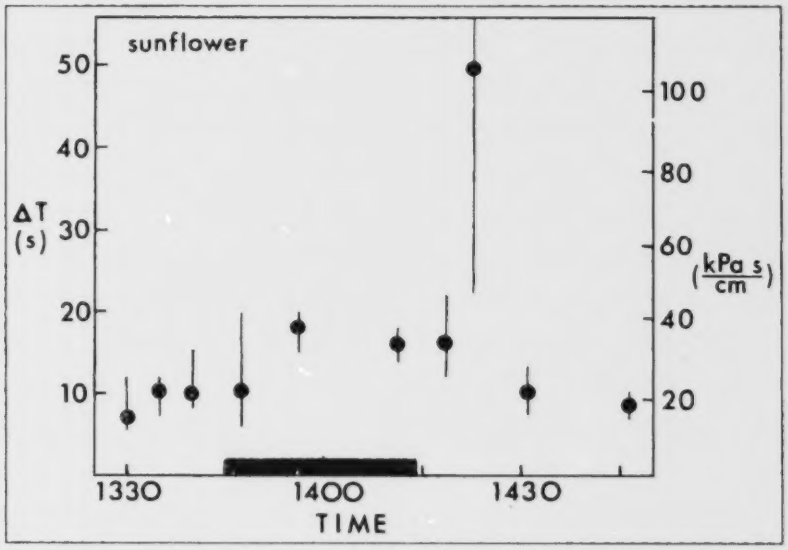

Figure 4. The response of the upper leaves of a sunflower to being moved from full sun to very low light (dark bar) and then back to full sun. Each point is the mean of four leaves and the bars show the range.

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Appendix

Units of Resistance

The rate of air movement across the leaf can be defined as a flux, Q, which describes the volume of air crossing a unit of leaf surface in a unit of time: $Q = \Delta V / A\ \Delta t$ (cm^3/cm^2s). It should be intuitively clear that the flux will be directly proportional to the pressure difference, ΔP(Pa), which drives it, just as with electrical current and voltage. Then $Q = \Delta P / R$ simply defines a leaf resistance, R, as the inverse proportionality coefficient that allows us to use an equal sign and have things come out right. Combining the above two equations, $R = \Delta P \Delta t A / \Delta V$ (kPa s/cm).

The thing to be careful about here is to remember that R is not the same as the more commonly used "stomatal resistance" (s/cm) which is based on the flux of water vapor from a leaf surface as measured by a diffusion porometer. Our resistance R is based on the mass flow of air across both leaf surfaces in series. In fact, there may be no simple relationship between the two measurements, even for a specific leaf, if the resistance of one leaf surface changes independently from the other, since the air flow porometer will always weight the higher resistance surface.

Plotting Rates of Photosynthesis as a Function of Light Quantity

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In this paper I discuss methods for plotting rates of photosynthesis as a function of light quantity. I recommend that the term "light intensity," which is physiologically irrelevant in photosynthesis, be avoided and that, if the appropriate equipment is available, rates should be plotted as a function of photon fluence rates. I present evidence that suggests that empirically derived conversion factors, which are used to convert foot candles to photon fluence rates, should be used with extreme caution. And finally, I suggest how rate data are best plotted if you do not have access to any kind of light meter.

Misuse of the Term Light Intensity

When referring to the influence of light on the rates of a variety of photosynthetic processes, many textbooks and published laboratory manuals state or imply that light intensity diminishes with distance from the source. Light intensity is defined as the rate of light emission from a light source and the units of measure are lumens per unit solid angle, or candelas (Nobel 1991). Since light intensity is a property of the light source itself, it is meaningless to equate light intensity with distance. In other words, if its emission is stable, the intensity of a light source remains unchanged regardless of the distance from which it is perceived. For this reason, the term light intensity in relationship to photosynthesis should be explained to your class and then avoided.

Quantum Units

In photosynthesis, there is a direct relationship between the number of molecules that are photochemically altered and the number of photons within the 400-700 nm wavelength range that are absorbed, regardless of the photon energy. Consequently, as has been pointed out before (Hershey 1991), the ideal measurement of light quantity for photosynthesis is micromoles of photons (400–700 nm) per meter squared per second (μM photons m^{-2} s^{-1}). The photon fluence rate (sometimes called photon flux density) in these units can be measured directly with a quantum sensor connected to a suitable meter (e.g. a Li-Cor LI-189 quantum/radiometer/photometer, Li-Cor, Inc., Lincoln, NE). Consequently, if such a meter is available, rates of photosynthesis should be plotted as a function of photon fluence rate.

Illuminance Measurements

Illuminance meters are often available in teaching laboratories. They give readings in foot candles or the S.I. unit, lux. Although, as explained elsewhere (Hershey 1991), illuminance measurements are not directly relevant to photosynthesis, illuminance units can be used in relative studies. It has been reported that illuminance readings can also be used to calculate approximate photon fluence rates by applying conversion factors that have been empirically derived for a variety of light sources (Thimijan & Heins 1983). If the light source is an incandescent bulb with a tungsten filament, it is claimed that an estimate of the photon fluence rate in μmol photons m^{-2} s^{-1} (400–700 nm wavelength range) can be obtained by dividing the lux value at a given distance by 50 (Table 3, Thimijan & Heins 1983). Therefore, if your meter reads in foot candles (10.8 lux = 1 foot candle), these readings can be converted by dividing them by

Table 1. Comparison of measured photon fluence rates with those from illuminance measurements for two light sources.

Source	*Distance from Source (cm)*	*Illuminance (foot candles)*	*Photon Fluence Rate Calculated (f.c. ÷ 4.6)*	*μM photons m^{-2} s^{-1}) Measured*
300-watt bulb	5	2800	608	905
(GTE Sylvania,	10	1600	347	462
Drummondville,	15	1040	226	267
Quebec)	20	738	160	174
	25	560	121	124
	30	440	95	93
	35	360	78	74
	40	295	64	61
	45	270	58	50
	50	235	51	42
100-watt bulb	5	2000	434	633
(GTE Sylvania)	10	1200	260	330
	15	720	156	195
	20	495	107	124
	25	345	75	82
	30	270	58	59
	35	205	44	45
	40	175	38	36
	45	145	31	29
	50	135	29	25

50/10.8 = 4.6. To check the effectiveness of this conversion factor, I have used an luminance meter (Model 756, Weston Electrical Instrument Corp., Newark, NJ) and a quantum sensor (LI-190SA, Li-Cor Inc., Lincoln, NE) to obtain readings in foot candles from a variety of incandescent bulbs at 5-cm increments from 5 to 50 cm. Two examples are shown in Table 1. The calculated photon fluence rates correspond well to the measured rates only at 30 cm from the lamp. With smaller distances, the calculated values underestimate the measured fluence rates by as much as 33%, while at greater distances the photon fluence rate is overestimated by as much as 21%. This conversion, therefore, cannot be recommended with confidence.

Distance from the Light Source

Where no meters are available to measure light quantity, students are frequently expected to plot rates of photosynthetic processes as a function of distance from the light source. Because the actual independent variable, photon fluence rate, is not directly proportional to distance from the lamp, this produces a misleading curve. Figure 1 shows a comparison of the rate of photoreduction of DCPIP by isolated barley chloroplasts plotted against both distance from the lamp (Figure 1c) and the photon fluence rate (Figure 1b). The actual relationship between photon fluence rate and the distance from the light source depends on the inverse square law. Therefore, the photon fluence rate at a distance from the light source is inversely proportional to the square of the distance from the source. Consequently, if photosynthetic rate experiments are conducted at different distances from a lamp, the photosynthetic rate can be effectively graphed by plotting the reciprocal of the natural logarithm of the distance from the lamp on the abscissa. As shown in Figure 1a, the curve obtained shows good correspondence with the curve plotted against measured photon fluence rate (Figure 1b). Although this method underestimates the photon fluence rate close to the lamp, probably because it fails to account for light reflected from the bench top, the most deviant values are found at photon fluence levels at which the experimental system is light-saturated and so do not affect the shape of the curve.

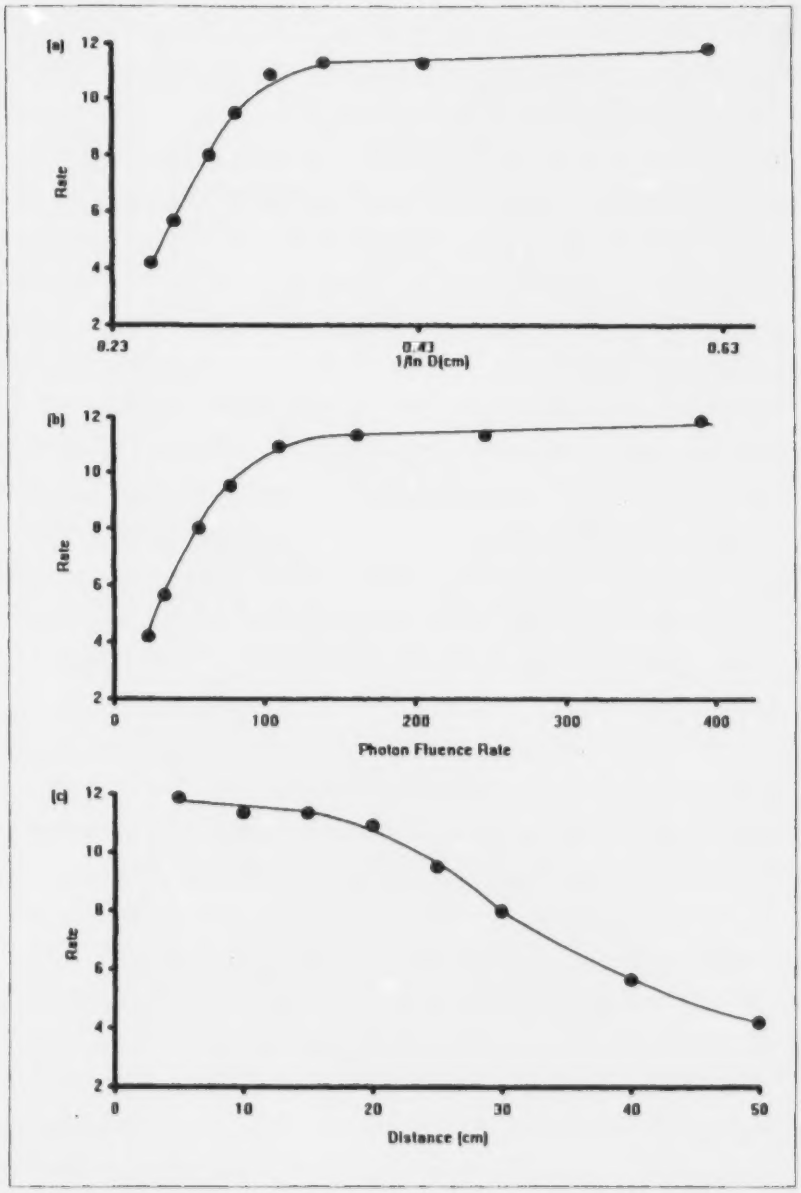

Figure 1. Comparison of plots of the rate of photoreduction of DCPIP by isolated barley chloroplasts (moles DCPIP reduced/minute/μg chlorophyll × 10^{-10}) as a function of: (a) The reciprocal of the natural logarithm of the distance from the lamp in centimeters (1/ln D). (b) The measured photon fluence rate (μmoles photons $m^{-2}s^{-1}$). (c) Distance from the lamp (cm). (Data from Dean 1996).

Conclusion

If your budget will accommodate a quantum sensor, this will provide the ideal units for the independent variable in the light-dependent steps of photosynthesis. Illuminance meters can be used for relative experiments or their units can be used to make crude estimates of photon fluence rates with reasonable accuracy over only a small part of the range. Plotting the reciprocal of the natural logarithm of the distance from the lamp produces

a curve that corresponds well to that plotted against the measured photon fluence rate. Finally, whatever unit of light quantity you choose to use on the abscissa, do not label this axis as light intensity!

Acknowledgment

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Animals

The Brain

User-Friendly & a Fascinating Classroom Subject

WARREN MARCHIONI, *THE MONTCLAIR KIMBERLEY ACADEMY, MONTCLAIR, NEW JERSEY*

The brain, according to geneticist and Nobel laureate, James Watson, is "the greatest challenge in science today." Watson's co-discoverer of the structure of DNA, Francis Crick, recently has published a book on how the physical brain is wholly responsible for what previously had been known as mind and all the remarkable phenomena associated with it—consciousness, memory, personality. More knowledge of the brain and how it works has been gained in the past 25 years than in all the time of study before then. Over the years, the brain has been referred to as a machine, a switchboard, a pump, a file cabinet, and more recently, a computer (Restak 1984). Obviously, each generation has its favorite analogy, depending upon whatever is at the cutting edge of technology at the time. Many believe that the human brain may be the most complex substance in the universe. It contains more than 10 billion cells or neurons and some trillion synaptic interconnections. The number of different possible mental states of the human brain would therefore be raised to this power; that is, multiplied by itself 10 trillion times. This unimaginably large number is far greater than the total number of elementary particles (protons and electrons) in the universe (Sagan 1977).

Davis (1984) has criticized the choice of outer space as the last frontier of knowledge. He doesn't believe that the center of the atom or the bottom of the deepest oceanic abyss should be considered for that honor. He feels that the last frontier is within us—1400 grams of convoluted nerve tissue encased in our skull—the human brain. It is a complex subject, but some of the mysteries are unraveling and the findings are fascinating. It was for these reasons, and the promise of solving the problems of Alzheimer's disease, MS, Parkinson's disease, addiction, depression and others too many to mention, that former President George Bush formally proclaimed the Decade of the Brain, 1990–1999.

The scope, of course, of brain study for young biology students has to be limited by its complexity and the amount of time available in the current curriculum, but the section of the course dealing with it can be as lively as the daily headlines, where, more and more frequently results of current research can be found. The advancements in brain science seem to compete on a nearly equal basis with those resulting from studies of DNA and AIDS, the other two headline-grabbing areas of current biological science.

The size of the human brain shows intricate variation among individuals, largely, but not always, dependent upon body size. Women, for example, tend to have smaller brains than men, which is not surprising since women

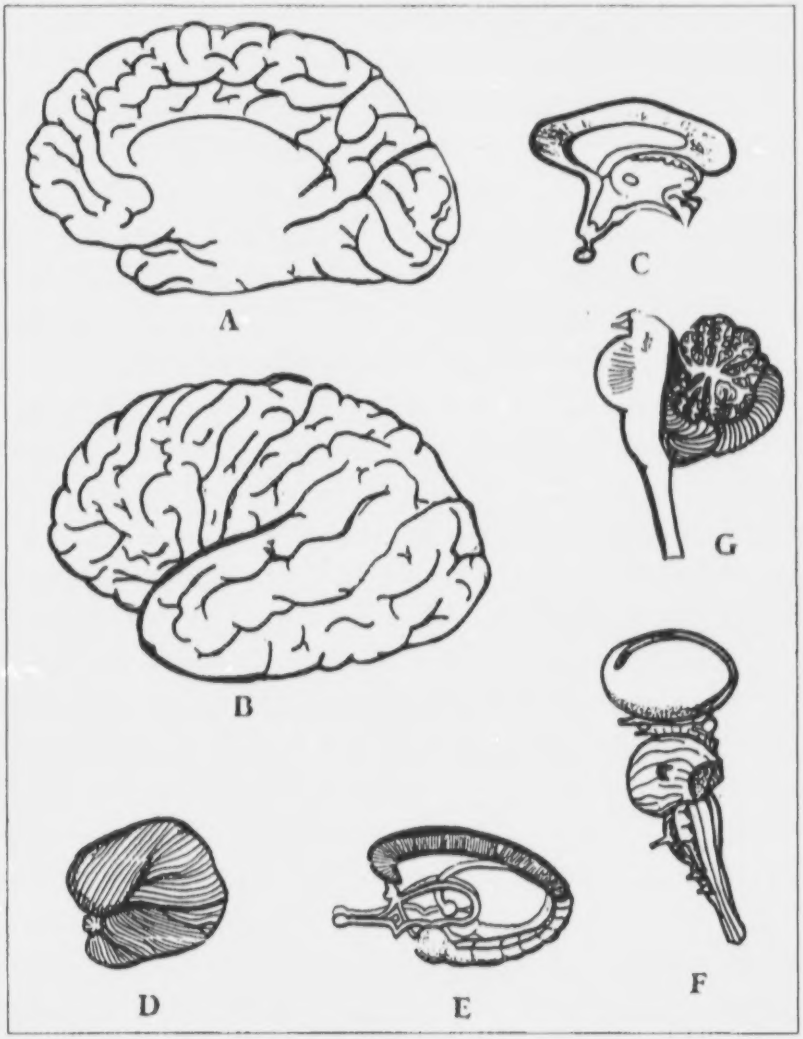

Figure 1. Template for construction of student 3-D model of the human brain.

tend to have smaller bodies than men. The brain of an adult male is about three pounds (1.4 kg). By capacity, this brain would fill a space of 1400 cc or about the volume equal to three pints of milk.

Brain size in humans has turned out to be a poor indicator of intelligence, as one of the largest brains ever measured was that of a severely retarded individual, while several creative and productive individuals in history have had relatively small brains for their body size. As examples, the American poet, Walt Whitman, possessed a brain of only 1282 grams and the French author, Anatole France, had a productive, but unimposing brain of 1017 grams. Attempts have been made in the past to correlate intelligence with brain size, and brain size with race and ethnicity. The techniques employed and the results obtained were motivated and influenced by the prejudice of the times during which these researchers worked (Gould 1981).

The human brain is an organ with many divisions, not all of them visible from the surface. For this reason, it is suggested that a better understanding by students of its anatomy will require them to construct a three-dimensional model. Figure 1 provides cut-out sections of a complete human brain. This template can be enlarged on a copying machine to approximate actual size. The template should be glued to thin cardboard and then cut carefully into the seven sections. The sections then can be layered into the correct positions according to Figure 2. Sections may be held together by a paper clip so that the model can be taken apart for study and review.

Based upon phylogenetic and embryological studies, the brain has been divided into three principal parts—forebrain, midbrain and hindbrain. The forebrain consists of the two prominent cerebral hemispheres (sections A and B) connected by the corpus collosum and deeper, not visible sections (C and F) that include the limbic system, basal ganglia, and diencephalon or "between brain." (The latter consists of the thalamus, hypothalamus, pituitary gland and pineal gland.) The small midbrain (a relay station for messages on their way to higher centers) occupies the uppermost part of the brain stem. The brain stem (pons and medulla oblongata) and the cerebellum make up the hindbrain (D and G).

For a more realistic view of the vertebrate brain, I discovered a reliable source of heads for brain dissections—

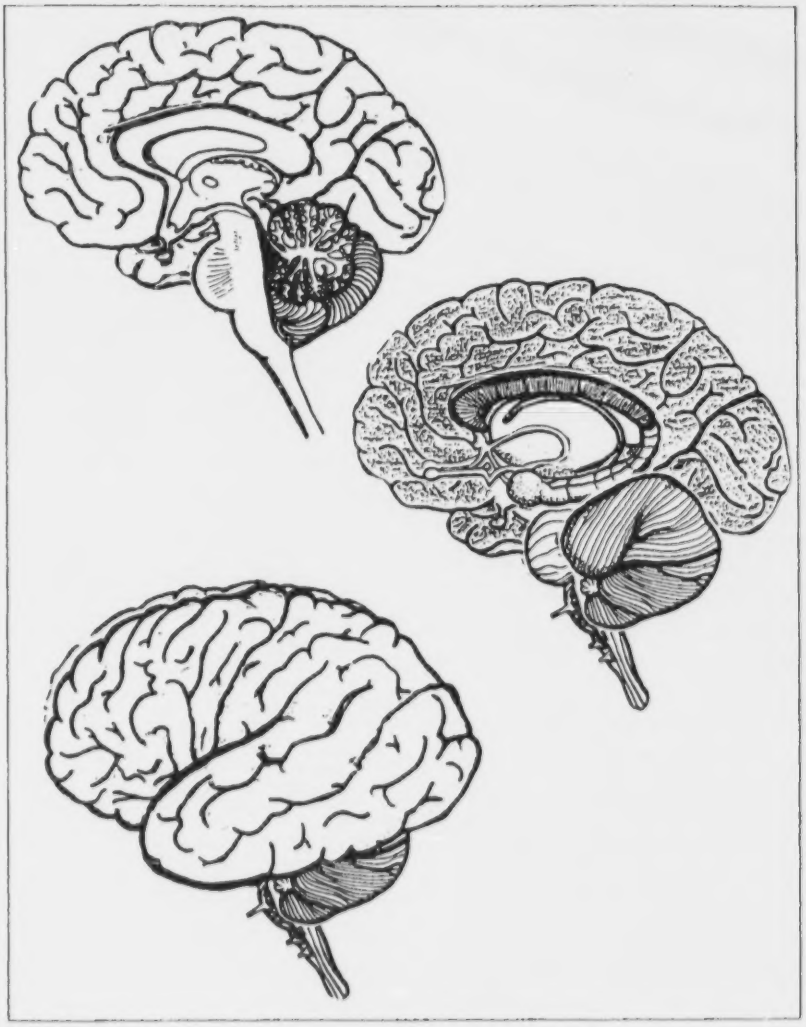

Figure 2. The brain model should be put together in the order shown here from top to bottom.

the local poultry dealer. The only part of the fowl that generally is not marketable is the head, and sufficient numbers for your students are available usually just for the asking. The heads should be preserved until needed to prevent any possibility of salmonella infection when they are used. However, long periods of preservation, particularly in alcohol, tend to harden the skulls, making the dissections difficult. Otherwise, the porous bone of the avian skull slices away easily and the brain can be extracted in one piece with meninges intact. Students, of course, should wear laboratory goggles and be instructed on the proper and safe methods of dissection. Figure 3 shows the position of the brain in the bird skull and the major components when removed.

As the brain is the most complex organ in the body, its development in the beginning of an individual starts early; for example, its rudiments can be seen in the embyro before the heart ever appears. One of the central problems in embryology is to understand how the billions of neurons can make the correct connections with each other. Cerebral neurons have many extensions, with a single nerve cell receiving as many as 100,000 different inputs (Wolpert 1991). From this intricate "wiring," programmed behavior is laid down in animals in fixed action patterns ranging from web building to navigation; the mental processes of

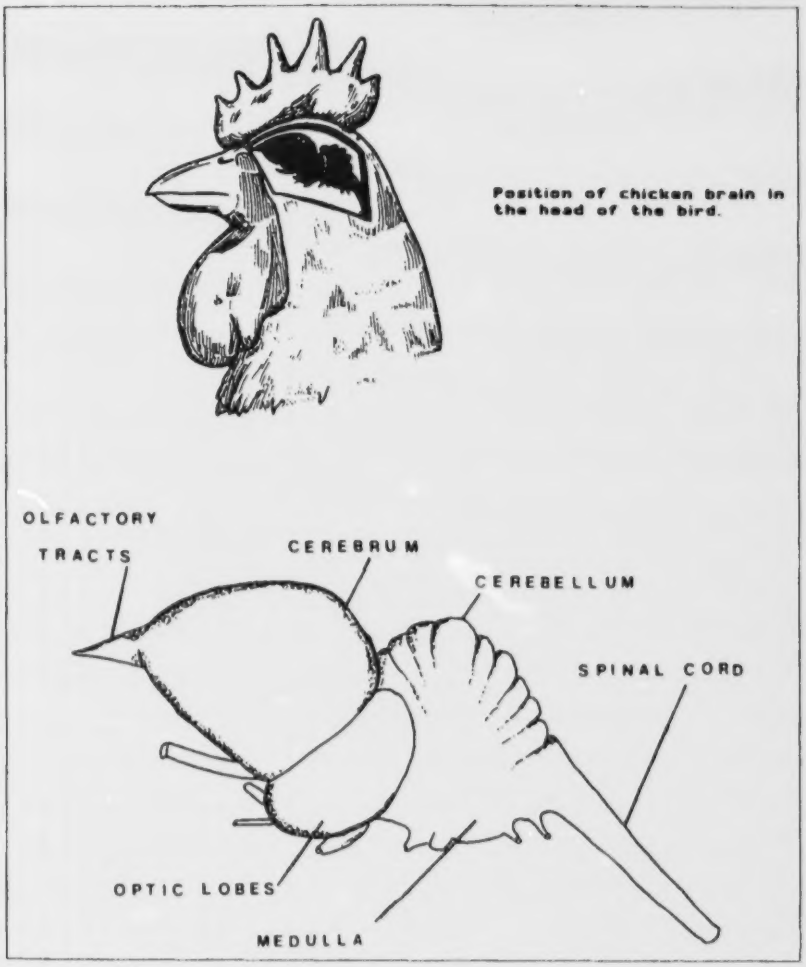

Figure 3. Position of brain in avian skull and sketch of the major external parts of the chicken brain.

consciousness, language and, perhaps, intelligence and creativity arise similarly in humans. Such possible correlations between human accomplishment and brain organization led to the request by scientists to examine Albert Einstein's brain after his death. Interestingly, the only unique feature that neuroanatomists could find was a greater than normal ratio of glial cells to neurons. Glial cells, which support and insulate the neurons, reproduce; neurons, with few exceptions, cannot.

The development of the central nervous system, which includes the brain and spinal cord, can be studied by using chick embryos. Sources of fertilized eggs can be found in most regions of the country or from biological supply companies. Incubation will be necessary only through the fourth day to demonstrate the marvel of vertebrate brain ontology. Eggs can be opened on a daily basis to demonstrate the progression from neural tube to head fold to the three-stage embryonic brain (proencephalon, mesencephalon, rhombencephalon) up to the final five-stage brain (telencephalon, diencephalon, mesencephalon, metencephalon, myelencephalon) (Figure 4).

Another approach to studying the brain in the classroom is to map a portion of the brain by testing the touch sense receptors of the skin. Human skin possesses several different sense receptors that can be differentiated from one another when a cross-section of skin is placed under the microscope. These different receptors respond to mechanical, chemical and thermal stimuli, allowing us to explore the characteristics of our environment.

Most of the activity in the cerebrum of the human brain is centered in a thin, convoluted surface layer only a few millimeters thick. Different regions of the cerebral cortex have different functions (Figure 5). For example, the somatosensory cortex straddles the foremost portion of the parietal lobe. Each point on this band of densely packed nerve cells represents sensory receptors from a different part of the body. Due to the crossing over of nerve tracts, the right half gets input from the left side of the body and vice versa. The specific amount of space in the brain designated to sensing each body part is proportional to the density of the sensory receptors in that area. As an example, the fewer the receptors in the upper arm, the smaller the upper arm sensory area is in the brain. Therefore, it is possible to map the entire body as it is "sensed," in this case through touch, by the cortex. Classically, this takes the form of a homunculus or "little man," which represents what a human would look like if built according to the sensitivity of body parts.

A device known as a sewing gauge, and calibrated both in the metric and English systems, is available from most sewing stores. This tool, with one fixed point and another one that slides along a calibrated scale, can be used to measure the distance between skin receptors. The class can be divided into teams consisting of an experimental subject (blindfolded) and an experimenter/recorder. Starting from the head and working down to the feet, each experimenter measures the distance between touch receptor fields in specific parts of the right hand side of the body, using the following procedure:

1. Spread the points apart and press the points lightly on the skin of the subject. The subject should detect two points of contact before preceding.
2. Move the two pins closer together, 0.5 cm at a time, until the subject no longer is able to distinguish two separate points. Measure this distance in cm. At this time, both points are within the same receptive field on one sensory receptor, and so the two points cannot be identified separately.
3. Repeat this measurement twice in the same general area. Average these measurements and record the mean in the data table (Figure 6).

Figure 4. Four-day-old chick embryo showing the five embryonic sections of the chick brain.

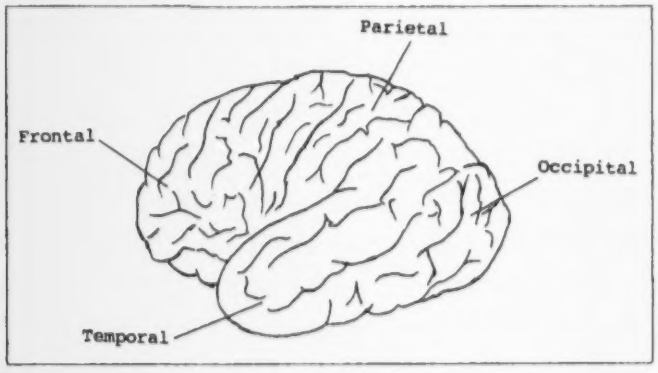

Figure 5. The human cerebral cortex with its four lobes: frontal, parietal, temporal and occipital.

4. Students should measure as many parts of the body listed on the data table as possible.

The number recorded for each body part represents the distance between each sensory receptor field, therefore the distance measured is inversely proportional to the left cortical area dedicated to that body part. That is, the closer the receptor fields, the larger the area represented on the cortex. To calculate the inverse, divide each mean into the number 1. For example: If the distance equals 0.2 cm, 1/0.2 divides out to be 5.0. Calculate the inverses for each body part and record them on the data table.

Students can then draw a proportional picture of a homunculus on graph paper. If the inverse is 5.0, then the body part occupies five boxes on the graph paper, approximating the normal body shape. To enlarge the scale, just multiply all values by the enlarging factor. For example, to make the drawing three times larger, multiply the inverses by 3. A typical homunculus created in my classroom is shown in Figure 7. While homunculi will vary due to the creativity of students, their proportions remain remarkably similar.

The nerve impulse is, of course, a complex transmission involving the flow of ions across the neuronal membrane and the production of neurotransmitters that bridge the synaptic gaps between neurons. This involved physiological action can be made more vivid to students through a simple, but measurable, activity that involves the whole class.

This activity can be done in the classroom or outside in good weather. Students should stand in a circle with their hands joined, leaving one break in the circle. The student to the teacher's left should hold a bicycle horn in his/her right hand. The teacher should hold a stopwatch in his/her left hand behind his/her back.

Simultaneously, the teacher squeezes the hand of the person to his/her right and starts the stopwatch. The student to the right then squeezes the hand of the person to his/her right, and so on around the circle until it reaches the last person, who immediately sounds the bicycle horn. The teacher stops the stopwatch the instant he or she hears the horn. Several practice runs should precede the actual experiment. By measuring the span between outstretched arms from tip of right hand to tip of left hand for several students in the class, an average distance the transmission traveled in one person can be determined. This length is multiplied times the number of people in the circle. By dividing by the time that elapsed, the speed of the transmission along large myelinated axons can be calculated. A comparison with laboratory data can be done by researching the literature on neurobiology. The differences can be discussed, bearing in mind the cerebral processes involved.

Through the activities described in this paper, students can learn about brain structure, development and function. They can also appreciate that this special organ of the body can be studied, directly and indirectly, by itself. For no other organ is this possible.

Acknowledgments

Thanks go to students Jeff Judge and Patrick Lindor for several of the illustrations used in the paper.

Sensitivity Data Chart

Site	Distance in cm	Inverse Values
Scalp		
Forehead		
Cheek		
Lips		
Tongue		
Chin		
Nose		
Neck		
Upper Arm		
Lower Arm		
Palm		
Elbow		
Back of Hand		
Fingertips		
Chest		
Back		
Abdomen		
Thigh		
Kneecap		
Calf		
Heel		
Sole		
Toes		

Figure 6. Table for recording data from measuring skin sensory fields.

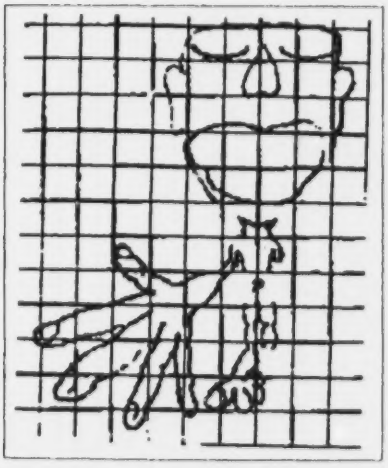

Figure 7. A typical sensory homunculus constructed by a student.

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Resources

Sewing gauge available from: Dritz Corporation, Spartanburg, SC.

BrainWork: The neuroscience newsletter is a very informative newsletter available free from the Eleanor Naylor Dana Institute, 1001 G Street, NW, Suite 1025, Washington, DC 20001.

Brain Facts is a primer on the brain and status of research in layman's language with illustrations. Available for $5 from the Society for Neuroscience, 11 Dupont Circle, NW, Suite 500, Washington, DC 20036.

The Brain, a highly acclaimed, eight-hour series on the brain, examining such topics as mental illness, stress, and the effect of drugs underlying sensation, perception, learning, emotions, and memory; and illustrating the brain's anatomy through state-of-the-art animated graphics. On ½" VHS videotape cassettes. From: The Annenburg/CPB Project, c/o Intellimation, P.O. Box 4069, Santa Barbara, CA 93140-4069.

Hologram of the skull/brain (3.4" × 3.4"). As you vary the viewing angle, the skull, with musculature, changes to reveal the brain and spinal cord. From: Arbor Scientific, P.O. Box 2750, Ann Arbor, MI 48106-2750. $15.

Measuring the Physical Properties of Silk From a Spider's Web

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Biomaterials are the building material used by nature to construct all living things. These biomaterials fill an enormous variety of functions, ranging from the stiff bones found in vertebrates that resist deformation during walking and running, to pliant arteries that deform to dampen pressure fluctuations generated by the heart. The structures of most of these biomaterials have evolved to maximize the performance of the function provided. In the process, nature has produced some biomaterials with physical properties that exceed or are comparable to some of the best man-made materials. The silks that form spiders' webs are an excellent example of these natural biomaterials. This article will describe how to measure the physical properties of the silks that form spiders' webs. Understanding how these natural materials function is becoming increasingly important for the design of superior synthetic polymers. The techniques described here have been used successfully to help explain how these natural materials function (Gosline et al. 1984).

Three physical properties of materials that are commonly compared are the modulus of elasticity, the strength of a material, and the energy required to break the material. The modulus of elasticity is a measure of the "stiffness" of the material and is defined later. The strength of a material is the "force" required to break it. However, engineers often take into account the size of the material when measuring these forces. This is easily accomplished by dividing force by the cross-sectional area of the sample; this ratio is called stress. Thus the strength is defined as the stress at which a material breaks. When measuring the strength of a material, engineers often plot the stress required to deform the sample against the amount that the sample is deformed. The latter is called the strain and is defined later. (The slope of this stress-strain graph is the modulus of elasticity.) The area under the line of the graph is the amount of energy needed to break the sample.

Table 1 provides some comparable data of spider silk and high tensile steel. Spider frame silk is about as strong as high tensile steel, but requires much more energy to break it. This is explained by the observation that spider frame silk is much more extensible than high tensile steel. More comparative data and further explanations of these observations can be found elsewhere (Gosline et al. 1986). The purpose of this exercise is to measure the modulus of elasticity of spider silk, using a micro-force transducer that can be built from materials readily available in the laboratory.

Procedure

Some care and attention should be given to the collection of the spider silk. Two different techniques can be used to collect the silk. First, take a small piece of cardboard (about the size of a playing card). Place a spider on the edge of the cardboard, and gently push it off. The spider will start producing dragline silk to prevent it from falling. Slowly rotate the cardboard so the silk wraps around it like thread on a spool. The second technique is simply to collect an entire web. This is easily accomplished with a piece of cardboard the size of the web. On the four edges of the board, arrange long pieces of tape so that the sticky side is out. Make sure that every edge has some tape on it. Now hold the board parallel to the intact web, and slowly push it through, so that the perimeter of the web becomes attached to the sticky tape. The students could be asked in advance to collect it, and during that process see how the web is built and how it functions. Simply store the webs in a clean area, such as inside a cardboard box, so that dust will not collect on the webs.

As described above, the stress required to deform the silk is very large. However, since the strand of silk is very small, the magnitude of the force required to deform it is also very small. This, of course, is obvious from the common experience of accidentally walking through a spider's web. To measure these very small forces it will be necessary to construct a micro-force transducer; one can be made from supplies available in any laboratory. The silk will be attached at right angles to the end of a very slender glass rod (the micro-force transducer). The principle of the transducer is that the strand of silk will be stretched and deform the rod. Simple principles from engineering can be used to calculate the force applied to the glass rod by the silk.

Now retrieve the cardboard that contains the dragline silk or the intact web. Individual pieces of silk now have to be removed from this "silk stock." The easiest way to accomplish this task is to cut a piece of cardboard

Table 1. Comparative values of the physical properties of spider silk and high tensile steel.*

Material	*Modulus* (N/m^2)	*Strength* (N/m^2)	*Energy to Break* (J/kg)
Spider Frame Silk	1×10^{10}	1×10^{9}	1×10^{6}
High Tensile Steel	2×10^{11}	2×10^{9}	1×10^{5}
Viscid Silk	3×10^{6}	5×10^{8}	1×10^{5}

* Data from Gosline et al. 1986.

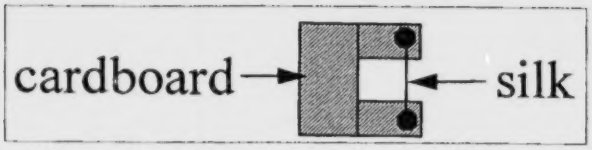

Figure 1. Cardboard apparatus used to isolate a single strand of silk.

in the shape shown in Figure 1. Attach a piece of tape on each end of the "U" so that the sticky side is out. Now carefully slide the paper under a single strand of web. This can be tricky, since it may be difficult to see ANY strands of silk. Sometimes this problem can be minimized if you arrange the light from a desk lamp so that it reflects off of the silk. Once the card is beneath the single strand, gently push the tape onto the strand, and with a pair of fine scissors cut the outer ends. You should now have a single strand of web suspended between the two ends of the "U." If you are removing a single strand from the intact web be careful that you know what type of silk strand you remove, since the physical properties will be quite different. Figure 2 shows the two types of silk that can be found in the web. The thin frame and radial strands are similar to the dragline silk described earlier, while the thick viscid silk has very different mechanical properties (Table 1). You are now ready to attach the silk strand to the mechanical test apparatus.

The test apparatus consists of a microscope slide, a cover slip, a thin glass rod, and some Vaseline™ (Figure 3). The glass rod can be produced in the lab by heating a solid glass rod over a Bunsen burner, and rapidly pulling it apart. A thin glass fiber will be produced between the two ends that were pulled apart. With some practice it is quite easy to produce a fiber that has a diameter roughly equal to that of a strand of hair. Now use the glass fiber to produce a delicate cantilever by gluing one end onto the top surface of one or two pieces of cover slip. Use five-minute epoxy for all of the test apparatus. Arrange it as shown in Figure 3a. Make a small mark (scratch) on the microscope slide close to and parallel to the end of the glass rod (or place a fixed marker such as a piece of dark tape). Now place a small quantity of Vaseline on the other end of the microscope slide, and place a cover slip on top of the Vaseline. The test apparatus is now complete and ready for attachment of the silk strand. Arrange the cardboard support shown in Figure 1 so that one end of the attached silk is directly over the end of the glass rod, and the other end is over the cover slip. Now carefully glue the two ends of the silk strand, making sure that the end of the glass rod is not glued to the microscope slide. After the glue has dried, cut the cardboard support away. If time permits, proceed to the next step. Otherwise store the whole apparatus in a clean area and use it to make the mechanical measurements during other class.

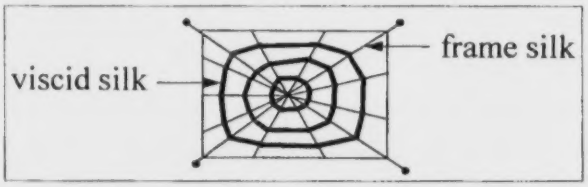

Figure 2. Schematic diagram of a spider's web. The web contains both frame silk (thin lines) and viscid silk (thick lines).

Two devices are required to measure the required parameters from the test apparatus. Calipers can be used to measure the change in length of silk strand and the length of the glass rod; but a calibrated stage micrometer of a compound microscope will be required to measure the deformation of the glass rod, and the diameter of both the glass rod and the silk strand. Measure the resting length of the silk strand (L_o in Figure 3a), and the length of the glass rod (l in Figure 3c). Using the compound microscope, measure the distance between the glass rod (where the silk strand is attached) and the marker beside it. This will eventually be used to calculate the deformation of the glass rod (a in Figure 3c). Now stretch the silk strand by sliding the cover slip over the Vaseline it is resting on. Stretch it sufficiently so there is a visible (and hence measurable) deformation of the glass rod. If you reach the end of the microscope slide before there is a measurable deformation, then the glass rod is probably too thick. Note that the Vaseline is viscous enough to overcome the force generated by the tension in the silk strand. Now place the apparatus on the microscope and measure the deformation of the glass rod (a in Figure 3c). (This is best accomplished by measuring the distance between the glass rod and the marker and subtracting the resting distance measured previously.) Measure the change in length (ΔL) of the silk strand. This is equal to the deformed (d in Figure 3b) minus the amount that the glass rod deformed (a in Figure 3c), or $\Delta L = d - a$.

Now calculate the strain on the silk strand, using the following relationship:

$$Strain = \frac{\Delta L}{L_o}$$

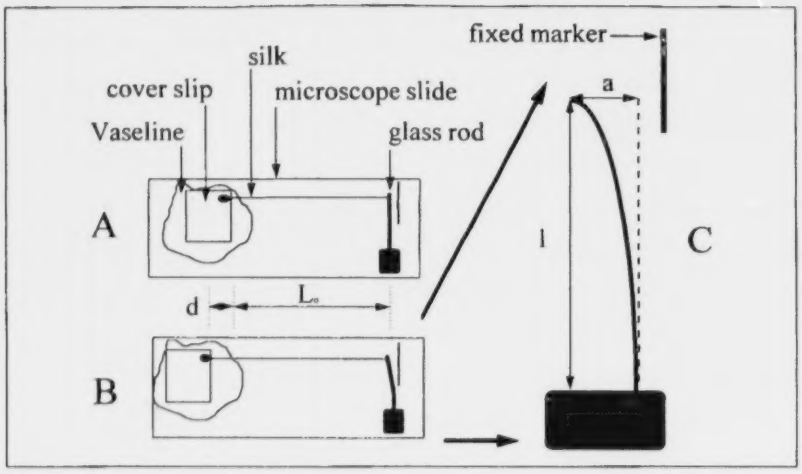

Figure 3. Apparatus used to measure the physical properties of silk from a spider's web. (A) The test apparatus consists of a microscope slide with a thin glass rod glued onto it, forming a miniature cantilever. The silk strand is glued onto the end of the glass rod and to a cover slip resting on Vaseline. (B). When a force is applied to the cover slip, the glass rod cantilever is bent. (C) The deformation of the glass rod (a) can be used to calculate the magnitude of the force applied by the stretched silk. This information can then be used to calculate the modulus of elasticity of the spider silk, as described in the text.

The force applied by the silk strand to deform the glass rod can be calculated from:

$$F = \frac{3aEI}{l^3}$$

where all of the parameters were measured above, except E, which is the modulus of elasticity of the glass itself, and is 7×10^{10} N/m^2, and I, which the moment of area for the glass rod and is calculated from:

$$I = \frac{\pi d^4}{64}$$

where d is the diameter of the glass rod. The stress can be calculated from:

$$Stress = \frac{F}{A}$$

where A is the cross-sectional area of the silk strand, calculated using the following equation:

$$A = \frac{\pi d^2}{4}$$

where d is the diameter of the silk strand. The modulus of elasticity of the silk strand is the ratio of stress to strain, and should be about 10^{10} N/m^2 if frame or dragline silk was used, or about 10^6 N/m^2 if viscid silk was used.

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Teaching Ecological Interactions with Mud Dauber Nests

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Ecological concepts such as food chains, communities and interactions between species are routinely covered in life science curricula at many grade levels. In my experience, students readily relate to simple food chains such as grasshoppers or rabbits eating vegetation and in turn being consumed by birds or foxes. However, concepts such as parasitism and nutrient cycling can be more difficult to grasp.

Over the past few years, I have used the distinctive nests of mud dauber wasps (Figure 1) as the basis for successful laboratory activities, both in university courses in ecology and behavior and in public school life science classes. The activities appear adaptable to a range of student ages and abilities, and seem to challenge and stimulate even the most complacent students.

Mud dauber nests are widely available, inexpensive and safe. They are easily collected and stored prior to use in the laboratory exercise. After describing, measuring and sketching the nest exterior, open the nests, identify the contents using a simple dichotomous key, and record the data. Two to three 45-minute laboratory sessions are usually required.

The unexpected presence of a variety of scavengers, parasites and predators in the nests offers students an engaging opportunity to gain firsthand appreciation of the diverse connections and authentic interactions among members of an unusual microcommunity. One of the objectives of this activity is to get students to ask questions that become progressively more scientific. Comparison of class results quickly moves beyond "Did I get the right answer?" to more scientifically meaningful questions such as, "What sample size is necessary to reflect the situation in nature?" Food chains and webs will differ from one nest to the next, illustrating the dynamic nature of these relationships.

The activities provide students an opportunity to develop and practice basic skills, from careful dissection to identification, observation, measurement and communication. The process closely resembles what a scientist does in that students can frame hypotheses, draw conclusions, and make inferences based on observations and collected information. Students' research into this topic is open-ended and real, conducted like that of working field biologists and potentially just as valuable to the scientific community. Even today, new discoveries about mud dauber nests are being described in the research literature (for examples, see Coward & Matthews 1995; Molumby 1995).

What Are Mud Daubers?

Mud daubers are medium-sized, non-aggressive wasps in the family Sphecidae. In the United States, three species are common (Figure 2): the organ pipe mud dauber (*Trypoxylon politum*), the black and yellow mud dauber (*Sceliphron caementarium*) and the steel blue mud dauber (*Chalybion californicum*).

The preferred species for this activity is the organ pipe mud dauber, found throughout the eastern United States, from southern Maine to central Kansas and southeastern Texas. If the organ pipe mud dauber is not readily available in your geographic area, with slight modifications one of the other species can be substituted. Both are found throughout the entire United States and southern Canada, and have been widely introduced elsewhere.

A Mud Dauber Life

Unlike the perennial hives of honeybees, the nests of mud daubers are solitary, once-a-year undertakings. Although a single favorable location may have many nests, these are not a colony. Rather, they simply reflect common attraction to a favorable spot. The following information is based on Shafer (1949) and Cross et al. (1975), plus the author's own experience.

In early to mid-summer, mud daubers emerge from last year's nests. After mating, females begin to construct nests near a reliable source of water or mud and abundant spiders. Organ pipe mud daubers and black and yellow mud daubers pick up mud in their jaws. The steel blue mud dauber, however, carries only water, which it uses to soften and remove mud from preexisting nests made by the other two species. After reusing others' nests, it reseals them with this recycled mud.

Each female organ pipe mud dauber begins by constructing a single mud tube, an activity that requires from three hours to a full day's labor. The female then switches to hunting spiders. Finding one, she stings it, then carries it back to the tube in her jaws and packs its paralyzed body inside. The wasp may gather from 3 to 18 spiders before she pauses. (The number of spiders appears to be related to their size, a matter both of spider species and of seasonality, for spiders get larger as the summer progresses.)Finally the wasp lays an egg across the abdomen of one of the last spiders, then seals off the prey and egg behind a mud partition. After the wasp has repeated this entire process two or three more times, making a series of cells within a tube, the entire pipe is full. Usually during construction the female is joined by a male who moves into the tube, mates repeatedly with her, and stands guard, defending the nest from insect intruders and competitors. Over the single season of her short adult life, a female mud dauber may construct several nests in various locations or add new tubes to preexisting nests. However, neither she nor her mate show any further interest in the completed nests or the offspring within. The other two mud dauber species have similar behavior, except for the presence of a guarding male.

Mud dauber development is an example of complete metamorphosis, in which the immature and adult forms are completely different creatures not unlike a caterpillar and butterfly. After about two days, the egg hatches into a young larva, which consumes the spider on which it rests. Moving freely about within its cell, the growing larva eats the remaining

Figure 1. The two common kinds of mud dauber wasp nests that occur widely in the United States. (left) The nest of the organ pipe mud dauber, *Trypoxylon politum*, consists of one or more parallel mud tubes typically 10–12 cm in length. Note the surface texturing, a series of chevron-shaped mud strips. The absence of large emergence holes indicates that this is a current year nest. (middle) The reverse of the same nest shows mud dividers that partition each tube into individual cells. (right) A representative nest of the black and yellow mud dauber, *Sceliphron caementarium* (maximum length 40 mm).

spiders over the course of about 10 days. In the now-empty compartment, it constructs a mesh cradle about itself, then fashions an elongate cylindrical cocoon (Figure 2), supported and suspended in the silk.

Inside the cocoon, the larva enters diapause, a cold-induced developmental delay. It remains a limp yellow prepupa for about nine months or for the duration of the winter. (In the southern United States, a part of the population may develop without diapause late in the summer to produce a small second generation of adults.) When warm temperatures return, development resumes. The larva molts into a pupa, acquires adult coloration gradually over the next six to eight weeks, then molts one last time into a winged adult wasp. Shortly afterward, the adult chews its way to freedom, first through the cocoon, then through the side wall of the nest. Interestingly, while larvae subsist on spider meat, adult mud daubers apparently feed upon nectar sipped from flowers, supplemented perhaps by the body fluids of spiders they have stung.

Obtaining & Storing Nests

Nests for classroom use should be collected during the cool months between September and April, when nests have been abandoned, and the nest contents are in diapause. You may wish to collect the nests yourself. Alternatively, students are often aware of places where mud dauber nests occur and are eager to help collect nests. Consider extra credit or other suitable rewards.

Bridges are particularly rewarding places to look for nests. Other good sites include old barns (look on the walls around entrances), golf course shelters near water holes, under decks and porches, and on carport walls.

Typically, favorable sites will have nests of various ages and conditions. If you have a choice, collect current nests for these activities. Old nests from previous nesting cycles characteristically have large holes in their sides where the new adults have chewed through to emerge. Sometimes a later occupant may have refilled these holes with mud of a different color. Current nests (Figure 1 left)—those completed during the most recent nesting season—lack these holes because the new generation of wasps is still inside. (Current nests may have an occasional small hole, however, if they have been parasitized.)

Nests attached to wood will be easier to remove than those attached to

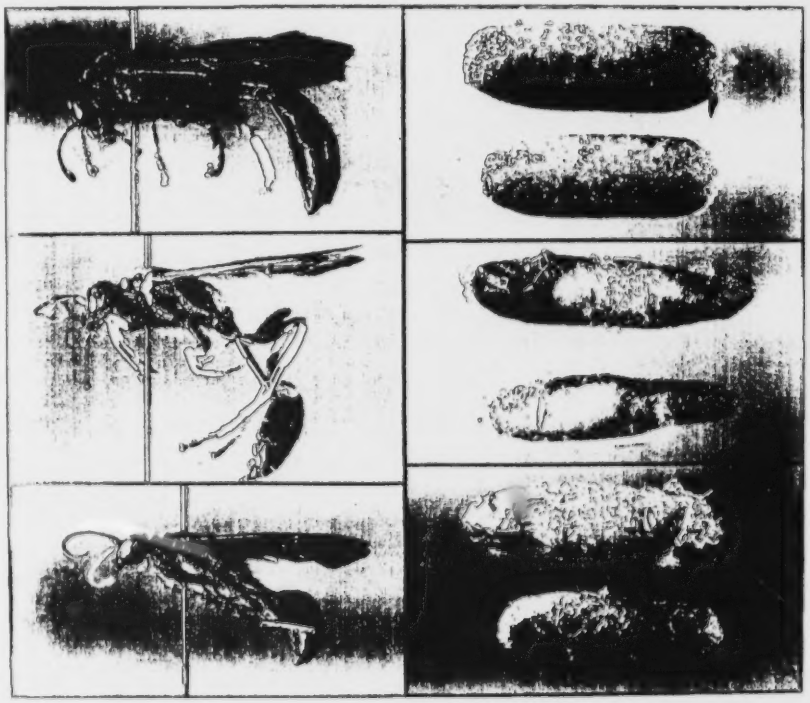

Figure 2. Adults and cocoons of the three common mud dauber wasps in the United States (top to bottom): *Trypoxylon politum, Sceliphron caementarium, Chalybion californicum*. Anterior (head) ends of cocoon face to left. Note bumps on head end of *T. politum* cocoons and the "chuck chamber" (at right end) of cocoons of *S. caementarium* that contain larval waste. Cocoons of *C. californicum* are more opaque and leathery compared to the flimsy translucent material characteristic of *S. caementarium* cocoons. Adults and cocoons measure 20–30 mm long.

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concrete. Use a putty knife to pry off and dislodge nests. As they fall, catch them in a net or box. Place each in a separate resealable plastic bag, labeled with the collection date and locality.

Store bags in a refrigerator until use. Like all arthropods, the nest inhabitants are ectothermic and can be kept refrigerated almost indefinitely.

Laboratory Planning & Preparation

Prior to conducting this activity with students, dissect a nest yourself. Identify the organisms with the aid of the dichotomous key (Figure 3), and save representative inhabitants for a class reference collection. For a durable, attractive and inexpensive display mount, place cotton batting inside a plastic petri dish or between a pair of clear food container lids such as those found on some yogurt containers. Center the nest inhabitant and an identifying label on the cotton, close the mount, and secure the sides with cellophane tape.

Most of your class will probably have seen a mud dauber nest at some point, and a few may have knocked them down. However, it's rare that a student knows what to expect inside the nest. To set the stage and heighten the suspense, weave an introductory scenario in which the students are scientists from Mars, travelers in the uncharted rain forest, or early North American explorers. They have come upon the mud structures for the first time and, knowing nothing about them, decide to carefully describe and dissect them.

When students first receive their nests, some will want to immediately break them open. Encourage students to work slowly and to use care to avoid breaking nests open during the exterior nest analysis. Attention to detail at this point will help them draw meaningful comparisons with the data they gather from inside the nest later.

After students have sketched and measured the external dimensions of the nest from both sides, the nests may be placed in labeled plastic bags and stored in the refrigerator until the next class session. Devote the second period to dissecting each cell and identifying and tabulating the contents. A third class period can be spent analyzing and discussing class results. The dissection activity is most appropriate for the middle and high school levels incorporated into units on ecology, but is adaptable to any level or time schedule.

Teacher Information

The flat side of the nest was attached to the building. Often the contents of the cell compartments are visible (Figure 1, middle), because the wasp uses the wall surface as the fourth side of her nest, conserving materials and energy.

Students should be encouraged to develop hypotheses to investigate. For example, one hypothesis might be that the parasitism rate is higher for larger nests than for smaller ones. Students may wish to compare their data with published measurements. In Alabama (Cross et al. 1975), the organ pipe mud dauber averaged 3.8 cell compartments per completed mud tube, with an inside tube width of 10.2 mm and average tube length of 106.2 mm (range 75–145 mm). The average cell length was 19.9 mm (range 7.4–28.0 mm). Interestingly, the mother wasp apparently constructed larger cells for her female-producing eggs: the average length of male-producing cells was 20.7 mm; for female-producing cells, 22.7 mm. Other sources of comparative data include Molumby (1995) for the organ pipe mud dauber and Shafer (1949) for the black and yellow mud dauber.

Each of the chevrons, about 1-mm wide, results from two trips for mud, applied to alternate sides of the tube. Different colors reflect more than one source of mud.

The main purpose of the nest is to protect the offspring from intruders and (since they are cannibalistic) from one another. Thicker walls might provide greater protection, but also might interfere with the ability of offspring to emerge. Physical properties of the construction material (mud, water and wasp saliva) probably also constrain the architectural possibilities. Students should be encouraged to do library research into animal architecture (i.e. bird nests, termite mounds) as an extension to this topic.

Holes in the nest wall, if present, may be of two sizes. The larger holes (2–5 mm diameter) spaced at about 20-mm intervals represent places where new adult offspring chewed out sideways to escape their cells. Occasional smaller holes (about 1 mm diameter) most likely were exits made by pirate flies.

Many factors affect the population dynamics at a particular locality. In a large sample of organ pipe mud dauber nests from nine localities in Missouri and Mississippi (Molumby 1995), mortality from all causes averaged 36% (range, 1–57%). The tiny parasitic wasp *Melittobia* was the major cause of larval mortality, parasitizing an average of 23% of all cells (range, 0–35%). Carpet beetles accounted for 7% of failures, cuckoo wasps and bee flies for 3% each, and pirate flies for an additional 2%. Velvet ants occurred at only three of the sampled locations. Each mother wasp constructed an average of 12.27 cells in her lifetime, and half of these failed. In an earlier study, Cross et al. (1975) obtained essentially similar results in Alabama.

In order for students to construct a meaningful food web, they will need to be introduced to food chains/webs, predators, parasites and scavengers. These terms will need to be understood in order to place the identified nest inhabitants in their proper food web positions. A typical web of interactions between mud dauber nest inhabitants is shown in Figure 4. In addition to the relationships shown, *Melittobia* may parasitize the other parasites, a phenomenon termed hyperparasitism. If the mud dauber's egg fails to hatch or dies, spiders may serve instead as food for scavengers such as carpet beetles and book lice, both of which feast on dried insect remains. However, pirate fly larvae can subsist only on freshly paralyzed spider prey; they deposit eggs or tiny larvae on the spider prey as it is being brought into the nest during the provisioning phase and somehow appropriate the prey for themselves, perhaps by destroying the mud dauber's egg.

How do your students' findings compare with those in the published literature? By keeping careful records and combining results of several classes and/or successive years into a master data set, your classes could obtain information of sufficient scientific value to merit publication. Data like those of Cross et al. (1975) and Molumby (1995) are valuable for comparative purposes, especially if obtained from new locations. Nesting habits of the organ pipe mud dauber vary with a number of environmental factors (see Barber & Matthews 1979; Brockmann 1980).

Expand upon this activity by trying to rear the various nest inhabitants to adulthood. Record information such as sex ratios, development times, degree of developmental synchrony, etc. These data are also potentially publishable. Another extension is to research the concept of biological control, and consider the implications of parasitism and predation on insect populations, both pest and beneficial. Additional information on mud daubers and their nest associates can be found

1a.	Object enclosed in a capsule or cocoon-like structure	go to number 2
1b.	Object not enclosed within a cocoon or capsule	go to number 4
2a.	Structure large, black, >10 mm long, filling most of a cell, often cradled in silken threads (Fig. 2) ⇨	Cocoon containing offspring of original builder, a mud dauber. (In very old nests cocoons may have one end broken and plastered over with mud. This means that a smaller inhabitant – probably a wasp or bee – subsequently used the old empty cocoon as a nest cavity.)
Note:		to further identify cocoon contents go to number 10
2b.	Structure smaller, <10 mm long; black or tan	go to number 3
3a.	Structure cylindrical, with rounded smooth ends (sometimes one end is broken open), usually loose in the cell among remains of spider parts; often several in same cell compartment ⇨	Pupa case of pirate fly (*Amobia* or *Senotania*), a pirate of the mud dauber's prey.
3b.	Structure leathery, with fuzzy cover; compact. not cylindrical. Often tightly stuck to corner of cell compartment ⇨	Cocoon of cuckoo wasp parasite (*Trichrysis*).
4a.	Six legs present	go to number 5
4b.	Eight legs present ⇨	Spider – If dead and dry, probably remains of original prey collected by mud dauber to feed its young; if alive, probably a recent arrival using mud nest as shelter.
5a.	Wings absent	go to number 6
5b.	Wings present	go to number 7
6a.	Very small, inconspicuous, less than 1 mm long. Crawls relatively quickly. Several often found together ⇨	Book louse (*Liposcelus*), a scavenger on nest leftovers.
6b.	Larger, bristly body with many hairs, especially at rear end. Often several of different sizes in a cell, together with old shed skins ⇨	Carpet beetle larva (*Trogoderma*), a scavenger that feeds on dried spiders.
7a.	Very small, black, about 1 mm long, gnat-like in appearance, often several dead in a single cell ⇨	*Melittobia*, a gregarious parasitic wasp.
7b.	Larger insects (>5 mm in length)	go to number 8
8a.	Bright metallic iridescent green or blue, 2 pairs of wings ⇨	Adult cuckoo wasp (*Trichrysis*), a parasite.
8b.	Dull black, sometimes marked with white. One pair of wings	go to number 9
9a.	Wings with distinctive black markings ⇨	Adult bee fly (*Anthrax*), a parasite.
9b.	Wings clear. Resembles an ordinary house fly ⇨	Adult pirate fly (*Amobia* or *Senotania*), a parasite.
10a.	Cocoon contents cannot be tipped out or easily dislodged; instead a tightly woven tan cocoon found within ⇨	Cocoon of velvet ant (*Sphaeropthalma*), that parasitizes prepupa inside cocoon. Careful examination of cocoon exterior may reveal a small mud spot where the velvet ant pierced the cocoon to deposit her egg within, then attempted to conceal her act.
10b.	Cocoon contents (or at least part of them) can be readily emptied onto a paper	go to number 11
11a.	Contents consist of a single large, legless, grub-like organism	go to number 12
11b.	Contents consist of numerous (>50) very small grayish legless grub-like organisms clumped tightly together ⇨	Immatures of a gregarious parasitic wasp, *Melittobia*, the most common parasite of mud dauber wasps everywhere. (Cocoons may contain only shed skins and a few dead adult *Melittobia* from an earlier attack (see number 7a)).
12a.	Grub body yellowish, very limp, with white spots ⇨	Immature mud dauber (prepupa) in arrested development for surviving the winter.
12b.	Grub body whitish, rather stiff, not at all limp ⇨	Overwintering larva of parasitic bee fly (*Anthrax*).

Figure 3. A key to the most common arthropod inhabitants of a current-year organ pipe mud dauber nest. Inhabitants of other kinds of mud dauber nests will be similar. Older nests may often have a significantly more diverse assemblage of squatters, including various solitary bees and wasps, ants, spiders, pseudoscorpions, and beetles.

in Matthews et al. (1996) from which this activity is adapted.

Safety Considerations

Are mud dauber nests safe to collect? Yes. Collecting nests during the cool months between September and April ensures that nests have been abandoned, and the nest contents are in diapause. However, if you should need to collect nests during the summer, there is little risk from their makers. Unlike paper wasps, mud daubers are solitary nesters and do not defend their nests, even while constructing them. If disturbed during active nesting, the wasps simply fly away. They are completely non-aggressive toward people.

Are mud dauber nests safe in the laboratory? Yes. Because of the wasp's life cycle, an adult would never lurk in a nest collected in fall, winter or spring. The spider prey, if still alive, cannot move except for an occasional leg twitch, for they have been stung and paralyzed. The insects within the cocoons are helpless grub-like immatures. Unless they are kept at room temperature for weeks, they will not complete their development.

Because nests contain various living organisms, including microbes, simple prudence dictates that you require students to wash their hands after handling nests.

Acknowledgments

I am grateful to the many middle school life science teachers throughout Georgia who participated enthusiastically in the workshops in which these ideas were developed and refined, and to the many students who have found these activities engaging and informative. Robin Hurst-March of the University of Missouri also provided valuable input. The National Science Foundation supported this work in part under grant ESI-9353040. Opinions expressed are those of the author and not necessarily those of NSF.

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Student Activity: What's Inside a Mud Dauber's Nest?

Mud dauber nests are familiar sights to most people. On the walls of buildings, they may persist for years. To the casual observer, they look like shelters made to live in. You may have noticed the maker—a solitary, non-aggressive wasp. However, had you stayed to watch her work for several days during the summer, you might have been surprised to find that as soon as the nest was finished, she flew off, and never returned.

If she's not going to live in it, why does a wasp go to all this trouble? In fact, the nest serves as a well-stocked nursery for her offspring. Within its walls, each of the wasp's young is presented with fresh meat to feed upon, separated from hungry siblings, and protected from enemies. How well does the system work? Careful research may provide an answer.

Materials

For each pair or small group of students:

- One mud dauber nest in a plastic resealable bag
- Individual laboratory notebooks
- A white paper plate
- One pair of forceps
- Dissecting needle or toothpick probe
- Approximately 6 vials or small jars with stoppers
- 10× magnifying lens or a dissecting microscope
- Marking pen to label containers
- Metric ruler
- One empty egg carton
- Copies of dichotomous key (Figure 3)

Procedures

1. Working in teams of two or three, assemble the materials listed above.
2. Open the bagged nest and place it carefully on the paper plate. Do all of your work on this plate, which will contain the dirt for later disposal.
3. Observe the exterior of the nest. Which side was attached to the substrate? In your notebook, describe the surface texture and colors of the mud. Which end faced up? How can you tell?
4. Trace the nest outline in your notebook. Measure and record the nest dimensions and the size and location of any holes. Find a section where textured mud arches or chevrons are clearly visible. Count the number of chevrons present along one centimeter of the tube's length. Multiply this number by the total length of the tube. What might each chevron represent? What does the total quantify? (Hint: Construction mud is brought one mouthful at a time.)
5. Is each tube a continuous tunnel? Turn the nest over. Observe and sketch the architectural structure as it appears from the flat side. Measure and record the dimensions of any visible compartments (called cells). Why would cells be needed? What functions might they serve?
6. Measure the thickness of the mud wall at five different points along the exterior wall, and obtain an average. Do the same for the cross-partitions between cells. Which is thicker? Why? What advantages might a thick exterior wall serve? What disadvantages might it have? What physical and biological factors might limit its thickness? Why might a relatively thin or nonexistent back wall be advantageous?

* suggested break between labs here*

7. With the marker, number the compartments in the egg carton. Then, beginning at one end of the nest, carefully use your fingers to break into the first nest compartment. Switch to the forceps to gently remove the cell contents. Place them into compartment number 1 in the egg carton. Repeat for each of the cells of your nest. How many cells did your nest contain? What are the dark cylindrical objects? Did every cell contain one?
8. In your notebook, construct a table in which to list and quantify the contents of each cell. Then use the key (Figure 3) to identify them. Do not open the cocoons at this time. Based on

the key, how many different organisms did your nest contain?

9. Examine the intact cocoons. Are both ends the same? Sketch and describe one in your notebook. Can you guess which is the anterior (head) end? Measure each one, and calculate the average cocoon length. Record the average and the range in cocoon sizes in your notebook. How might this be different if the nests had no cells? What other factors might cause sizes to vary?
10. Do all of the cocoons really contain mud daubers? Grasp a cocoon in your fingers and hold it under the hand lens or microscope. Examine it carefully for anything unusual, such as a tiny hole or a spot of mud. Then use your fingernail to carefully break off one end of the cocoon. Gently tip out the contents onto a piece of paper. Use the key to identify the contents. Repeat for each cocoon. Record your results in your notebook.
11. Construct a web or concept map (Figure 4) that depicts the relationships between each of the organisms you found. For extra credit, use library or classroom references to research the habits of the different nest inhabitants.

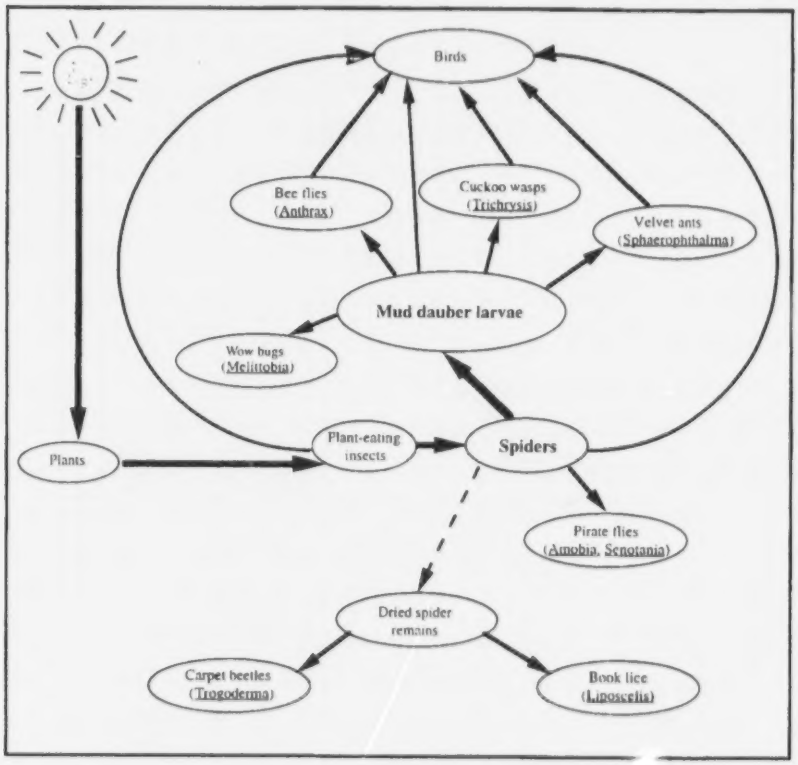

Figure 4. The food web associated with the organ pipe mud dauber nest habitat (arrows show the direction of energy flow). Because both spiders, and mud dauber wasps are predators, at least two trophic levels exist outside of the nest habitat. Producers (plants) and consumers (herbivores) underpin the entire micro-community found in the nest.

Additional Discussion Questions

1. Many of your nests had various holes in the mud walls. Was there any relationship between hole size and cell contents? Explain.
2. What percentage of the cells yielded living mud dauber offspring? What, in descending order, were the causes of larval mortality in your nest? How did overall class results compare to yours? What does this suggest about mud dauber nesting strategies?
3. Suppose a female mud dauber can make 12 cells in her lifetime. Based on your own nest, how many offspring would survive to become part of next year's population? Compare your results with those of others in the class. What similarities can you find? How might you account for the differences?

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An Improved Model for Demonstrating The Mechanism of Breathing

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Problems with the Bell Jar Model

The bell jar model (Figure 1) is commonly used in biology lessons to show the mechanism of diaphragm action in ventilation. In this model, the balloons represent the lungs, while the bell jar and the rubber sheet stand for the rib cage and diaphragm, respectively. When the rubber sheet is moved up and down, the balloons in the bell jar become deflated and then inflated. This model is useful in helping pupils to visualize the action of the diaphragm in breathing. It has, however, some features that may lead to a misunderstanding of the mechanism of ventilation.

Below is a typical textbook passage describing how the model works (Yip 1992, p. 150):

> *When the rubber sheet is pushed upwards into a dome shape, the balloons become deflated. This is because the air volume inside the bell jar decreases, thus increasing the air pressure. As a result, the volume of the balloons decreases and the pressure increases. Air is forced out of the balloons.*
>
> *When the pressure on the rubber sheet is released, it becomes flat. This increases the air volume inside the bell jar, so decreasing the air pressure. As a result, the volume of the balloons increases and the air pressure inside them decreases. Air is sucked into the balloons.*

The problem with this explanation lies in the fact that the bell jar is filled with air, but the pleural cavity is actually filled with a serous fluid. For the bell jar model, the air inside the bell jar expands and contracts according to the movement of the rubber sheet, and this then leads to changes in the size of the balloons. Quite different from this, however, the fluid inside the pleural cavity of the thorax is not compressible. As a result, downward movement of the diaphragm leads directly to an increase in lung volume, and this decreases the air pressure inside the lungs. The reverse occurs when the diaphragm moves upwards. This mechanism of diaphragm action differs from that described for the bell jar model in textbooks. By explaining changes in lung volume in terms of fluctuations in air pressure inside the bell jar, an inaccurate and erroneous picture of the mechanism of ventilation may be developed by students, and this may interfere with further understanding of the ventilation mechanism at a more advanced level of study.

Another problem with the model is that as air is highly compressible, a large movement of the rubber sheet leads only to a relatively small change in the size of the balloons. This actually simulates the situation of pneumothorax that occurs when some air is trapped inside the pleural cavity. People with this condition cannot ventilate their lungs efficiently even though their diaphragm can work normally. On the other hand, as the pleural fluid is incompressible or inexpansible, changes in volume of the thoracic cavity in a normal lung caused by diaphragm action will be directly translated into volume changes of the lung.

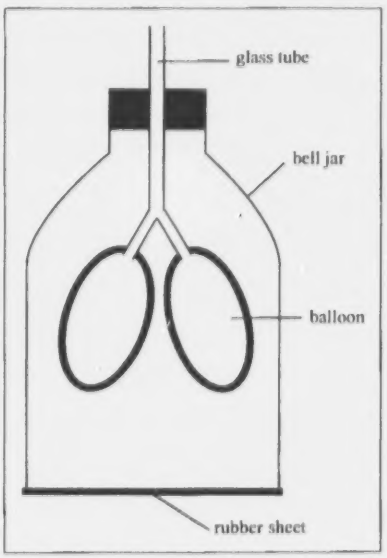

Figure 1. The bell jar model of the thorax.

An Improved Design of the Bell Jar Model

The above limitations can be overcome by a modified design of the original bell jar model. Figure 2 shows such a novel homemade model. A 500-ml plastic bottle (e.g., a soft drink container), preferably with a strong, rigid bottom, is used. The base of the bottle is cut away with a pair of scissors. The rubber sheet at the bottom, simulating the diaphragm, is prepared by cutting a large balloon; it is fitted tightly to the base of the bottle using a metal ring. A small balloon, standing for the lung, is fixed to the glass tube on the rubber stopper with a rubber band. The bottle is filled with water instead of air, representing the pleural fluid. The small balloon is filled with some lead shots (or water) to prevent it from being buoyed up by the surrounding water.

By filling the bottle with water instead of air, the movement of the rubber sheet gives a better simulation of the action of the diaphragm during normal breathing. As water is incompressible, movement of the rubber sheet can be directly translated into changes in the volume of the balloon inside the bottle. The changes in balloon size produced by the movement of the rubber sheet in this design is much more spectacular than that of the air-filled bell jar model. This is illustrated in Figure 3.

As the wall of the plastic bottle is flexible, squeezing the bottle wall and releasing the pressure can demonstrate the effects of movement of the rib cage. These actions cause the balloon inside the bottle to become deflated and inflated, respectively.

The apparatus can also be used to demonstrate the effect of a pneumothorax. Releasing the clip on the rubber tubing causes the balloon to collapse immediately, and the balloon can no longer be inflated by whatever movement of the rubber sheet (Figure 4). This condition provides a vivid simulation of the situation when the pleural membrane is punctured (Vander et al.

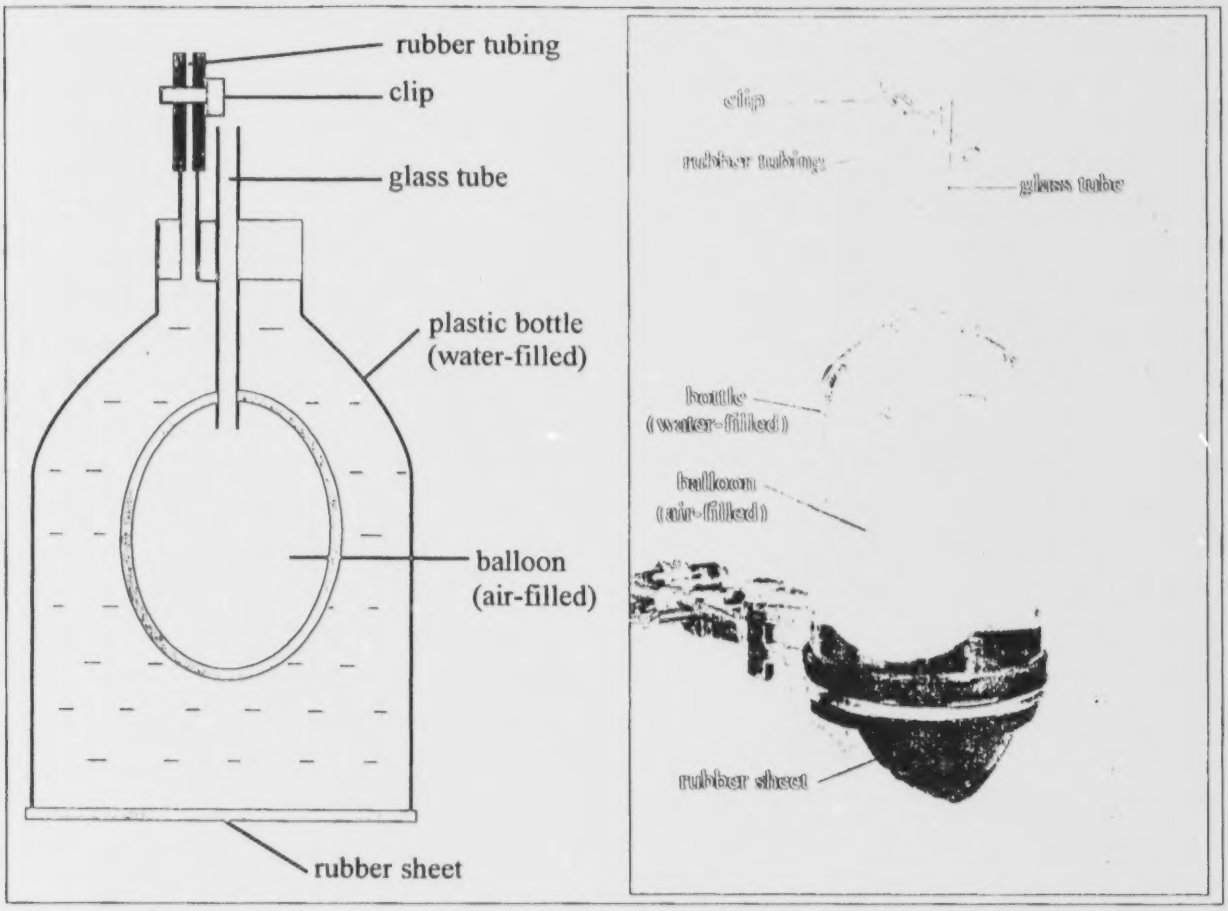

Figure 2. A novel design of the bell jar model.

1994, p. 479). This model can be easily constructed by pupils themselves using simple, readily available materials which include:

- A 500-ml plastic soft drink bottle
- A small balloon to represent the lung
- A large balloon for preparing the rubber sheet
- A rubber stopper fitted with glass tubes
- A metal ring for fixing the rubber sheet to the base of the bottle

Through such an activity, not only can pupils develop a more direct and accurate understanding of the concept of ventilation, they will also become more motivated, creative, and involved in the study of biology.

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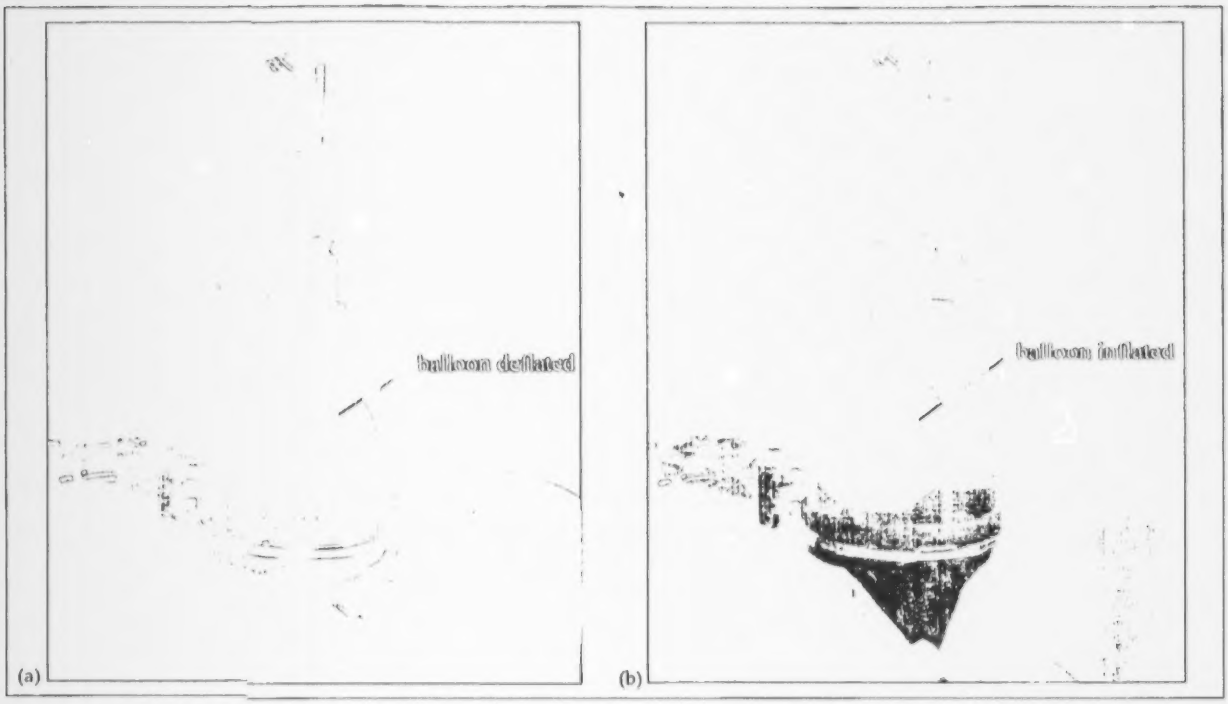

Figure 3. Effect of movement of the rubber sheet. (a) Rubber sheet moved upward. (b) Rubber sheet moved downward.

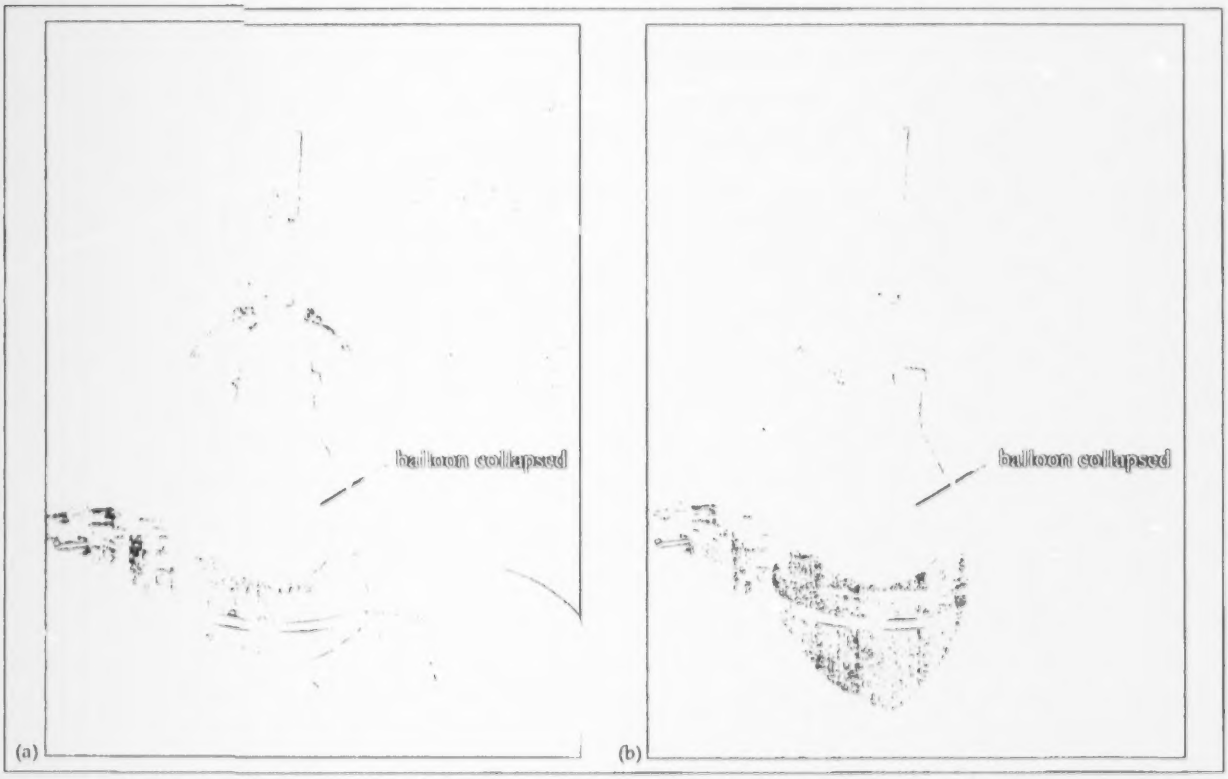

Figure 4. Model simulating a punctured pleural membrane. (a) Rubber sheet moved upward. (b) Rubber sheet moved downward.

Drosophila Fly Anesthetic

A New Technique

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In these scientifically advanced times, it is surprisingly hard to find a good *Drosophila* fly anesthetic for lab or classroom genetics experiments. Many anesthetics have been used, such as ether, CO_2 and Fly Nap®. Ether is effective, but it is flammable and highly toxic. CO_2 is effective, but there hasn't been a good way to apply it. Fly Nap is not flammable and it is easily applicable. However, the smell is objectionable to some humans and it can be expensive to buy. On the other hand, dry ice is an inexpensive way to obtain ample amounts of CO_2. This gas is easily applicable, and it is cost effective.

Procedure

1. At your local ice cream shop or grocery store, purchase about one pound of dry ice per 1300 to 1500 fruit flies that will be anesthetized. Keep the dry ice in a styrofoam container until the anesthetization occurs.
2. Place a small pyrex funnel, with the tube facing downwards, in a *Drosophila* container with about 40 flies in it. None of the flies will escape through the funnel.
3. You may want to start anesthetizing in each jar in 5- to 10-minute increments to allow enough time to inspect the flies. Crush the ice into small pieces of about 8 cubic centimeters with an ice pick. Wearing thick gloves, insert a piece of dry ice into the funnel and cover the top. The CO_2 will sink to the bottom of the jar and spread evenly, because CO_2 is heavier than air. You may need to lightly tap the bottom of the jar on a table to settle the flies at the location of the CO_2.
4. When the dry ice is almost small enough to fall through the funnel tube, remove the funnel and cork the jar. Begin to take flies out of the jar in increments of 5 or 10 at a time by lightly tapping the jar on a table and examining them. This will continue anesthetization because the CO_2 will not escape from the jar.
5. Immediately after removing each increment, continue to cork the jar with the anesthetized flies in it. After each fly in the increment has been inspected, put the *Drosophila* in a separate jar from the ones that have not yet been inspected.

This process of anesthetization will take about 8 minutes. The fruit flies will remain anesthetized for about 13 minutes. Remember, if you think you'll use 5 pounds of ice, you might want to buy 7 pounds because the dry ice will continue to sublimate in the container. The length of the anesthetic effect will differ if the number of fruit flies in the jar is changed. The ratio of dry ice mass to the length of anesthetic effect is 8 cubic centimeters to 11 minutes of exposure per 40 *Drosophila*. When anesthetized for 20 minutes, the fruit fly mortality rate is approximately one per 40 flies (see Figure 1).

Acknowledgments

The author thanks Michael Neil for his thoughts and priceless input on ideas for anesthetics that involve carbon dioxide. The author also thanks Larry Hillman for his assistance on experimentation.

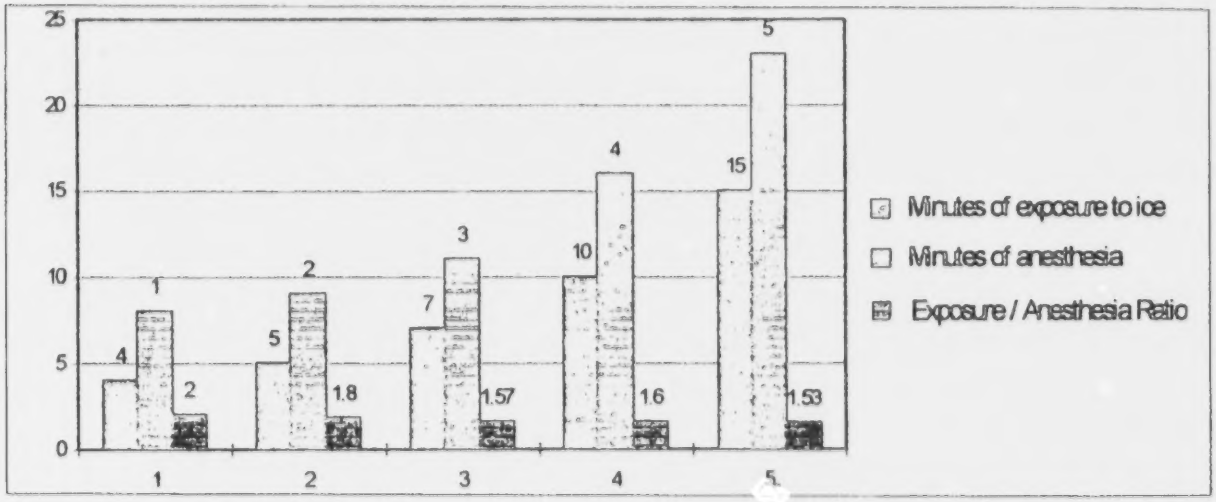

Figure 1. Results of ice exposure time.

Thermal Acclimation in Ectotherms

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Physiological ecology has contributed much to our understanding of adaptation to the physical environment, both among humans (Ingram 1975) and other organisms (e.g. Kavaler 1981; Alsher 1990). Yet it receives relatively little attention in high school or college-level introductory biology laboratories, probably because most experiments in this area of biology require sophisticated and expensive equipment. Here I describe a laboratory exercise on acclimation that is simple, reliable and inexpensive.

Acclimation is the process by which organisms adjust their cellular and physiological systems to cope with changes in the physical environment. Most organisms have an inherent range of conditions (a range of temperatures, for example) that are optimal for them (i.e. they will stay within this range as long as food and other requirements are met). In nature, however, environmental conditions change frequently. Acclimation is the process by which an organism adjusts its metabolism to cope with a changing environment.

This exercise examines the response of an aquatic ectotherm to changes in water temperature. In such organisms, body temperature is nearly the same as water temperature. If an animal that is acclimated to 20° C water is transferred to 30° C water, then the short-term response is a rise in metabolic rate as its body temperature increases. In most cases, a 10° C increase in environmental temperature will produce a two- to three-fold increase in metabolic rate over a span of hours; the exact magnitude of change is called the organism's Q_{10} (Schmidt-Nielsen 1979). Left in the 30° C environment for a few days, the animal's metabolic rate will decrease gradually until it returns to the previous level. At that point, it has fully acclimated.

Step 1: Preparing for the Experiment

In this exercise, ramhorn snails (*Helisoma* spp.) are placed in 10° C, 20° C and 30° C aquaria and given time to acclimate. Then, the snails are moved between aquaria to contrast the short-term and longer-term (acclimation) responses to temperature change.

About a week before the experiment is to be done, three aquaria are set up with differing water temperatures; 20° C is near room temperature, and the other temperatures can be reached by refrigerating one aquarium and using tropical fish heaters in the other. About 20 snails are placed in each tank. Ramhorn snails are available at pet stores and from biological supply houses, and can live on a diet of lettuce. Other snail taxa would probably work just as well, but I have not tried them. I have used goldfish (*Carassius auratus*) in similar experiments, but with less consistent results.

Step 2: Practicing the Winkler Test

The metabolic rate of a snail is measured by enclosing the animal in a water-filled vial; an identical vial without the snail is used as a control. The depletion of dissolved oxygen in the treatment vial as compared to the oxygen content of the control vial is a direct measure of metabolic rate in ml of O_2 consumed/hour. I use vials with a volume that is about 10 times greater than the volume of a typical snail. Within an hour, the snail will deplete the oxygen of the treatment vial to a measurable extent. If snails must be left in the vials overnight, larger vials (about 100 times the volume of the snail) can be used.

To measure dissolved oxygen, I use a version of the Winkler test with 10 ml water samples that have been drawn into plastic syringes. Details of the method are available in Dolphin (1992) or Drew & Robertson (1974), and are summarized in the Appendix. Hach Chemical sells a Micro Winkler Unit that can be used as well. The Winkler test requires precise technique to be accurate. Thus, I have students in groups of two practice the procedure four or five times. Each group practices with water samples taken from the same aquarium; usually, their precision improves. In addition, groups may compare their results and note that cold water has a higher dissolved oxygen content than warm water.

Step 3: Setting Up the Experiment

The experimental design is illustrated in Figure 1.There are five treatments; in each, two equal-sized vials are tied together with a rubber band. One vial contains a snail and water from the aquarium where it was housed, and the other contains the water only. In Treatments 1–3, metabolic rates are measured for snails that are kept in their orginal conditions. In Treatment 4, snails from the 10° C tank are placed in the 20° C tank; and in Treatment 5, snails are transferred from 30° C to 20° C. In larger classes, transfers from the 20° C tank to the other tanks can also be done.

I usually have students work in teams, with each team carrying out all five treatments. Vials are left in place for one hour, after which the oxygen consumption for each snail is determined. Because it takes about 10 minutes to complete a single run of the Winkler test, some snails are kept in the vials longer than others. To compensate for this bias, I instruct students to calculate metabolic rates on a per-hour basis. During the hour between setting up the experiment and doing the first Winkler test, the students propose hypotheses and identify the appropriate comparisons for testing them. For example, if the animals have acclimated as expected, the metabolic rates should be about the same for Treatments 1–3. Snails that have been transferred to a warmer temperature (Treatment 4) should have higher metabolic rates than those that remained in their original aquaria (Treatment 1). Similarly, snails that were moved to a cooler temperature (Treatment 5) should have lower metabolic rates than those that remained at 30° C (Treatment 3).

In my opinion, experiments used in teaching too often give "pat" results, i.e. results that are almost guaranteed to conform to students' expectations. This is handly a realistic representation of science; therefore I occasionally set up an experiment so that at least one

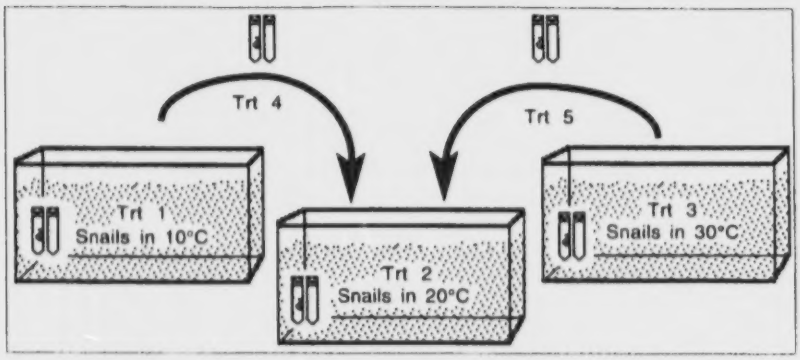

Figure 1. The experimental design involves five treatments (Trt). Snails are transferred to different temperatures in two of them.

finding is unexpected, then challenge the students to explain it without using the familiar appeal to shoddy laboratory techniques. In this experiment, we have consistently found that the snails are unable to acclimate to the 10° C environment. This challenges students to think about their findings and their assumptions in designing the experiment.

Typical Results

Figure 2 shows the results from one run of the experiment in which eight pairs of students participated. The median values show that the snails acclimated in Treatments 2 and 3, but not in Treatment 1. Statistical analyses confirm one's visual interpretation of the data: Mann-Whitney U-tests show that Treatments 2 and 3 are not significantly different, while Treatment 1 differs from both of them ($P < 0.05$). To explain this, students usually suggest that the 10° C water temperature is beyond the lower limit for acclimation in this species. Another possibility is that acclimation takes longer when the snails are moved to a cooler temperature than when they are moved to a warmer one, which Schmidt-Nielson (1979) suggests is a general phenomenon.

The short-term responses to temperature changes are as predicted. Snails moved from the 10° C tank to 20° C experienced a two-time increase in metabolic rate, while those transferred from 30° C to 20° C had a two-fold drop in metabolic rate. Both of these differences are statistically significant ($P < 0.05$).

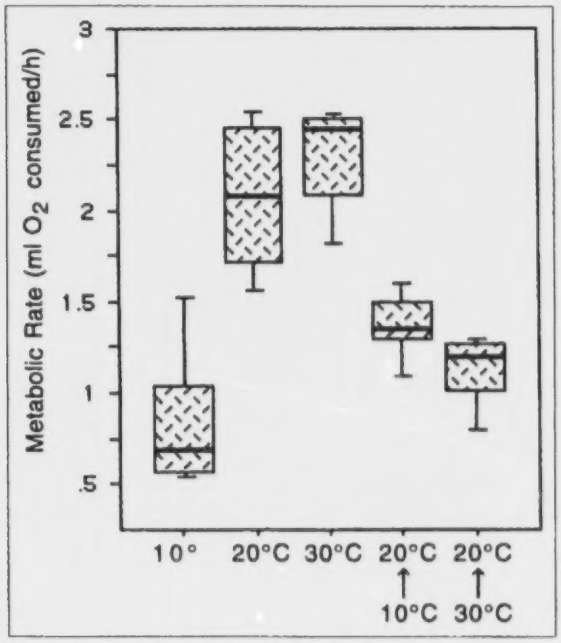

Figure 2. Boxplots of the results found by one class. The thick horizontal lines are the medians for each treatment. The shaded boxes enclose the 25th to 75th percentiles, and the vertical lines extend from the 5th to the 95th percentiles.

Summing Up

After we examine the results of the experiment and attempt to explain anomalous results, I ask the class to speculate on how acclimation is achieved. What changes occur in cells to enable the organism as a whole to cope with the temperature change? The scientific literature indicates that acclimation may be achieved at the cellular level by:

1. Production of a host of enzymes that are more efficient at the new temperature, or
2. Keeping the same enzymes, but changing their abundances.

Cells in colder climates require a greater number of enzymes if the rate of metabolism is to be constant. Among ectotherms, the second mechanism is most common (Hazel 1989).

My students rarely hit upon the right answers at first, and sometimes not at all. Their ideas are as colorful and varied as their personalities; and while many of their ideas may be incorrect, they are still valuable products of critical thinking. Creative hypothesizing is a key feature of good science.

Acknowledgments

My idea for this exercise grew out of two experiments that I taught as a laboratory teaching assistant at Iowa State University. The data presented in Figure 2 were collected by my ecology class at Emory University during the fall of 1989.

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Notes

Ecology & Evolution

Using Soil Seed Banks To Bring Plant Communities into the Classroom

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Teaching plant ecology concepts in high school and introductory college biology courses is often limited to readings and lectures due to logistical problems with field work. Time for travel and vegetation analysis in the field often prevent the study of plant populations and communities during scheduled class time. An alternative to studying plant community composition and dynamics *in situ* is to collect soil samples containing buried seeds (the "seed bank"), germinate the seeds in the laboratory, and analyze community dynamics through the composition of the seed bank. Not only does this technique overcome many of the logistic problems, but it provides a unique insight into the dynamics of plant communities.

The seed banks are composed of buried dormant but viable seeds. Some of the seeds may germinate immediately, but often a proportion of the seeds produced in a season will remain buried in a dormant state and will germinate intermittently over a long period of time. The seeds represent species currently growing aboveground, species that existed in the area in past years, and seeds that have been transported to the area. Each seed species has its own germination requirements and will only germinate when those specific conditions are met. Therefore, seeds may germinate and be recruited into the aboveground community when seasonal conditions are met (Baskin & Baskin 1985) or when a disturbance creates the proper conditions (Miles 1979; Grubb 1977). The seed bank also provides a source of genetic novelty for plant species (Levin 1990). Thus, each community has a unique seed bank that is an important factor in determining its specific vegetative composition (Fenner 1985).

Among the early investigators who noted that soils contain large numbers of viable seeds was Charles Darwin. Darwin recorded 537 seeds germinating in 210 g dry weight of mud (1859). Since Darwin's early observations, soils of many communities have been sampled and viable seed numbers estimated. The estimates of seed numbers vary according to plant community and methods of testing, but Fenner (1985) provides the following ranges for seed density:

forest soils	10^2–10^3 seeds/m^2
grasslands	10^3–10^6 seeds/m^2
arable soils	10^3–10^5 seeds/m^2

The dynamics of seed banks have proven difficult to study, especially with small seeds, due to difficulties in monitoring the fate of seeds. However, a generalized model (Figure 1) has been proposed by Harper (1977).

Investigation of these soil seed banks combines a relatively brief field experience (describing the ecosystem and collecting the soil samples) with later laboratory observation and analysis of the germinating seeds. This is a simple, inexpensive method that avoids significant habitat destruction, but allows students to make careful observations and to test a variety of student-generated hypotheses in community ecology.

Sampling Soil Seed Banks

Several methods have been used to detect the presence of buried viable seeds and to examine the floristic potential of seed banks (Roberts 1981). All of the methods involve either removing blocks of soil or using a soil corer to extract a soil core.

A simple method that I have found useful for undergraduate investigative laboratories is to prepare a metal cylinder by cutting the top and bottom out of a metal can. (Two- or three-pound coffee cans work well—the exact volume can be measured to give quantitative data on seeds/volume of soil.) This cylinder is then pushed into the soil until the soil is approximately 2–3 cm from the top of the can. In harder soils a board may be placed horizontally on top of the can and a person may stand on the board to drive the can into the ground. A shovel is then passed underneath the buried can and lifted straight up to remove the intact soil sample (Figure 2). To keep the soil in the can, the plastic top of a coffee can, a shallow pan or even heavy duty aluminum foil is now placed on the bottom to hold the soil in the can.

Any seedlings or plants present at the time of sampling may be recorded and removed or left in the sample, depending upon the experimental design. However, any disturbance of the sample at this point may affect which seeds germinate.

How many samples are collected will depend on experimental design, and limitations of time and greenhouse space. A large number of samples may be required to identify all species present, while smaller samples may be sufficient to identify community differences. To assure random samples, the sampling points may be taken along a transect. (For details of how to set up a transect, see a field ecology manual such as Brewer & McCann 1982.)

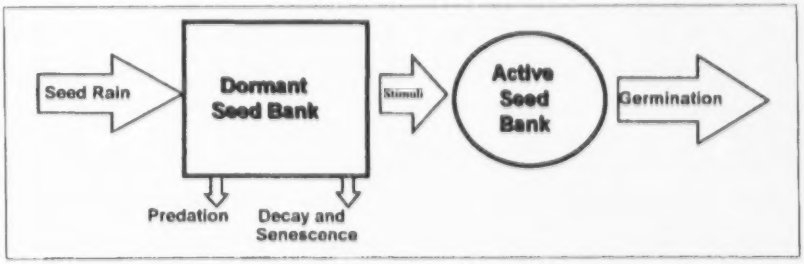

Figure 1. Flow chart for the dynamics of the population of seeds in the soil (Harper 1977).

Figure 2. Removal of soil seed bank sample.

The soil samples are taken back to the laboratory or greenhouse where germination trials are carried out in the same cylinders used to collect the sample (or the soil in the cylinders may be spread out over sterilized soil in shallow pans). Ideally, the seeds should be germinated in an environmental chamber, but most biology laboratories and greenhouses allow adequate control of environmental conditions (broad spectrum fluorescent lights and 21–30° C works well). The soil should be watered daily as the new seedlings are very susceptible to desiccation.

Any single germination regime will not trigger germination of all the species in a seed bank since the germination requirements of all species cannot be met with a single set of conditions (Major & Pyott 1966; Thompson & Grime 1979; Van der Valk & Davis 1978; Roberts 1981; Forcella 1984; Hopkins & Parker 1984). It should also be noted that lab and field conditions will be extremely different, and the germination of seeds in the lab will exclude a wide variety of both biological and physical factors that affect the seeds in the field. Some of the factors missing from the lab studies will include seed predators, seedling herbivores and extremes in climate.

If germination of all species is necessary (it may not be necessary for studies demonstrating differences in specific groups of plants), each sample may be divided into 3 aliquots and treated as follows before germination:

- 1 aliquot kept at room temperature
- The second refrigerated for 3–4 weeks
- The third heated to 38–40° C for 30 minutes.

However, dilatory or delayed germination may still not occur for one or two years with some species (Wilhelm & Mengler 1988).

A potential source of error is airborne seed ("seed rain") contamination of the sample. An adequate control would be to observe germination in sterile soil samples incubated in the same locality as the other samples.

For many studies (e.g. comparing species totals between two seed banks), the seedlings need not be classified but are simply identified as different from other seedlings (e.g. seedling "A" has round leaves, seedling "B" has hairy leaves, etc.). If specific identification of the seedlings is required, sketches, photographs and pressed specimens may later aid identification of the seedlings. For many parts of the United States seedling identification manuals are available (e.g. Chancellor 1966; Priwer et al. 1980; University of Georgia 1978).

Suggestions for Seed Bank Investigations

The following list contains suggested studies and relevant literature for student investigations:

1. Compare the composition of aboveground vegetation with the seed bank composition to determine the contribution of seed banks to aboveground plant communities (Bartolome 1979; Donelan & Thompson 1980; Leck 1980; Leck & Graveline 1979; Livingston & Allessio 1968; Major & Pyott 1966; Oosting & Humphreys 1940; Schiffman & Johnson 1992; Thompson & Grime 1979; van der Valk & Davis 1978; Whipple 1978).
2. Compare seed banks of cleared and/or burned areas with uncleared/unburned areas (Archibold 1979; Lamont & Barker 1988; Morgan & Neuenschwander 1988; Mueggler 1956; Zammit & Zedler 1988).
3. Examine possible allelopathic effects of leachates on seed germination (Groves & Anderson 1981).
4. Compare different layers of soil to determine differences in seed bank composition and density at different depths (Coffin & Lauenroth 1989; Henderson et al. 1988; Moore & Wein 1977).
5. Compare seed banks along an environmental or successional gradient (Jerling 1983; Young & Evans 1975).
6. Compare seed banks from cultivated (or grazed) land and uncultivated (or ungrazed) land (Brenchley & Warington 1933, 1945; Cavers & Benoit 1987, Champness & Morris 1948; Conn et al. 1984; Hume & Archibold 1986; Kinucan & Smeins 1992; Roberts 1981; Roberts & Neilson 1982).
7. Compare the effect of different weed control treatments on the seed banks (Hill et al. 1989).
8. Examine seasonal variation in seed banks (Thompson & Grime 1979; Baskin & Baskin 1985).
9. Examine germination factors such as light and moisture availability to determine environmental tolerances (Wesson & Waring 1967; Thompson et al. 1977).
10. Compare soil seed bank with "seed rain" (Rabinowitz 1981; Rabinowitz & Rapp 1980).
11. Compare seed banks of disturbed vegetation with undisturbed areas (Janik & Stearns 1987; Smith & Kadlec 1985).
12. Examine the effect of the presence or absence of seed predators (Louda 1987).
13. Examine the practicality of using seed banks for the (re)establishment of native plant communities (Johnson & Anderson 1986; Putwain & Gillham 1990; Wade 1987).

Few studies examining the soil seed banks of highly impacted areas, such as those with toxic metals, high UV radiation, acid rain, etc., have been published. However, student investigations in this area could be used to relate plant community ecology to current environmental issues and concerns.

Summary

An investigation using soil seed banks may be done as a group to demonstrate the concept of soil seed banks followed by individual investigations where students may develop their own hypotheses to test. This technique combines field study with careful laboratory observation allowing the student the opportunity to correlate what happens in nature with laboratory observations. An understanding

of plant community dynamics, an experience with the scientific process, and an increased awareness of local ecosystems are realistic outcomes of this type of investigative laboratory.

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Demonstrating Natural Selection Using Magnetobacteria

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Two of the things most highly valued in the science classroom are solid theories and laboratory experiences to test those theories. Natural selection is an important theory taught in any biology course and yet it is very difficult to find interesting and appropriate experiments to test it. Simulations abound involving yarn or beans or paper disks or toothpicks, etc. Such models can be very effective yet should never replace experimentation with nature itself. Most textbooks describe experiments that have demonstrated the power of selective pressures on a living population. Most involve eukaryotes with relatively long generation times. Such experiments often require years, if not decades, to complete.

Magnetobacteria offer the science teacher an alternative to decade-long experiments, colored beans, and counting colonies of yeast while peering through condensation. These dynamic prokaryotes have short generation times and are easy to maintain. They can be found in the sediments of many lakes, ponds, bays and marshes throughout the world. After I discuss some of the biology of magnetobacteria, I will describe how they can be used in an empirical test of natural seletion that can be performed successfully over a period of three to four weeks.

About Magnetobacteria

In 1975, Richard Blakemore was investigating marine bacteria collected near Wood's Hole, Massachusetts. While viewing live motile forms he observed that they had a tendency to collect at one edge of the hanging droplet. Regardless of the position of the microscope, the lighting, etc., the bacteria accumulated at the same edge. Eventually Blakemore realized that it was the northernmost edge of the droplet to which the bacteria swam (Blakemore & Frankel 1981). Other samples from both fresh and saltwater sediments contained similar north-seeking varieties. Later research revealed south-seeking bacteria in sediments collected in the southern hemisphere (Blakemore, Frankel & Kalmijn 1980).

Many types of magnetobacteria have been discovered in a variety of locations. Knabel (1997) recently discovered as many as 50 different species at a single location in Iowa. Typically found in undisturbed, unpolluted marine or freshwater sediments, they may be cocci, bacilli or spirilla. Some even exist as multicelled aggregates. The distinguishing characteristics that all magnetobacteria share is the presence of "magnetosomes," tiny membrane-bound particles of magnetite (Fe_3O_4), greigite (Fe_3S_4), or pyrrhotite. Each magnetosome is precisely made by the bacteria at a size of 400 to 800 angstroms to ensure the magnetic stability of the particle.

Within the bacterium, the magnetosomes are generally arranged in a single file line running parallel to the longitudinal axis. The number of particles can be anywhere from eight to more than 100. The line of magnetosomes serves as a compass needle, orienting the bacterium so that its longitudinal axis lies parallel with the Earth's magnetic field lines (see Figure 1). Student observation of bacillus or spirillum forms will reveal that the interaction between the line of magnetosomes and the Earth's magnetic field is *not* strong enough to propel the bacteria, only to align an already moving cell's axis with the field lines. Movement is seen only in living cells by the use of flagella.

Magnetobacteria are microaerophiles requiring near-anaerobic conditions.

Regardless of the species, populations found in the northern hemisphere are composed almost exclusively of "north-seeking" individuals. When sediment around these north-seeking bacteria is disturbed, their "internal compasses" (made up of magnetosomes) realign the swimming microbes with the Earth's magnetic field lines. These magnetic field lines are not parallel to the Earth's surface (except at the magnetic equator) but decline into the Earth with increasing angles at the higher latitudes (see Figure 2). Because the axes of the bacteria are realigned with the Earth's magnetic field, any forward movement by the north-seeking varieties will move the bacteria northward (at a downward angle), enabling them to rebury themselves in the near anaerobic environment that they require. If a south-seeking bacterium existed within the disturbed population, it would swim to the south (at an upward angle), away from the anaerobic sediments. In the southern hemisphere, however, this same south-seeking bacterium would swim forward at a downward angle and would thus reach the anaerobic sediments. Selection pressures, therefore, act against bacteria that seek the "wrong" pole.

Obtaining & Viewing Magnetobacteria

The collection and use of magnetobacteria has previously been described in detail by Guilfoile (1985). To obtain a classroom culture of magnetotactic bacteria, collect a sample of sediment in glass or plastic containers from locations where the water (freshwater or saltwater) is relatively calm and unpolluted. Possible sites include lakes, marshes, ponds, sewage treatment ponds, etc. Once a sample has been collected, loosen the lid and store the sample in a dark location. No other care or treatment is required. Sediment samples will typically increase in magnetobacteria density for several months, and if sufficient organic material exists, can support a population without maintenance for more than a year. If you cannot locate a natural source for these microbes in your area, a culture of *Aquaspirillum magnetotacticum* is available from Ward's.

To view the magnetobacteria you will need to create a hanging drop. To do this:

1. Place a ¾-inch rubber O-ring on a clean glass slide.
2. Using a dropper, obtain a small sample from just below the surface

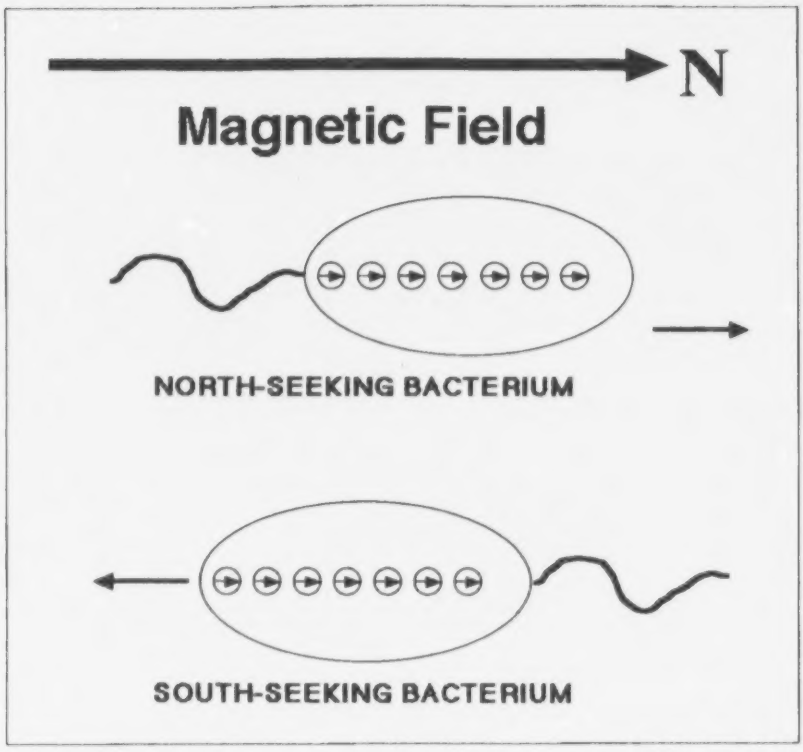

Figure 1. A line of magnetosomes within each of these bacteria acts as an internal compass needle, aligning the axis of the microbes with the magnetic force field lines.

of the sediment (where the sand and slit layers interface). Often, as your samples mature, dark bands will develop at this location (Knabel 1997).

3. Place a drop of the water/sediment mixture on a clean GLASS *coverslip*. Use the dropper tip to make the drop elongated. Creating a narrow "cove" on either end of the droplet greatly enhances viewing, as the bacteria will become concentrated in the cove.
4. Invert the coverslip using a circular motion (creating a hanging drop) and place it on the O-ring so that the drop hangs freely inside the O-ring.
5. Using reduced light, focus on one *edge* of the droplet (100X).
6. Place the *south* end of a small bar magnet (the end sought by the north end of a compass needle) *nearest the edge of the droplet on which you are focused.* If magnetobacteria are present, within a few moments you will begin to see the bacteria congregate *up against the edge* of the droplet nearest the magnet. Once the bacteria appear, it is helpful to use a higher power objective.
7. Pivot the magnet around so that the *north* end of the magnet (the end sought by the south end of a compass needle) is nearest the droplet edge on which you are focused. This reversal of magnetic force field lines will spin the magnetobacteria 180 degrees, causing them to swim toward the opposite side of the droplet. If any south-seeking individuals are present in the hanging drop they will eventually begin to accumulate at this edge.

Since most samples will contain very few south-seeking bacteria, it is helpful to demonstrate to students that the north-seeking variety is not the only one possible. This can be done by using a standard tape demagnetizer (tape eraser, tape degausser) available from an electronics store. Following the instructions on the demagnetizer, hold the device over a slide containing a droplet of north-seeking bacteria. The demagnetizer randomizes the orientation of individual magnetosomes within the bacteria's "internal compass needle." Once the demagnetizer is removed, the line of magnetosomes will become magnetically realigned with each other in order to achieve the lowest state of free energy. Since they randomly realign, about one-half of the bacteria will be north-seeking with the remainder being south-seeking.

Using Magnetobacteria To Teach Natural Selection

Magnetobacteria offer an excellent opportunity to perform a wet lab demonstrating natural selection. By using one of the following strategies your students can observe selective pressures forcing adaptation within a population. The goal is to create an environment in which north-seeking bacteria are not favored but are selected against.

One way to reverse the magnetic field within a jar is by using an electric coil. To do this, lay a small jar on its side and lay a compass inside it so that the needle swings freely. Wrap a meter or more of wire around the jar in one direction. Connect the wire terminals to a power supply or 6V battery while you watch the compass needle inside the jar. If the north-seeking end of the compass needle points to the open mouth of the jar, you are ready to proceed. If the north-seeking end of the compass needle points to the bottom of the jar, switch the terminal connections. As little as 0.5 amps of current running through a coil arranged in this way will create a magnetic field within the jar in which magnetic north is vertically upward and magnetic south is vertically downward. In such an environment, north-seeking bacteria will swim upward, away from the near-anaerobic sediments, and therefore will be selected against.

A second way to create a reversed magnetic environment within a jar is to use a magnet. Small flat magnets available in hobby and craft stores work well for this setup. Keep in mind that the goal is to create an environment in which north-seeking magnetobacteria will swim upwards and south-seekers downward. The Earth's magnetic field is quite subtle and can be negated by even weak bar magnets. Hold a magnet next to a compass to determine which side of the magnet attracts the north-seeking end of the needle. Mark this side of the magnet. Next, attach the magnet to the underside of the lid of the jar (use glue or a metal lid) so that the marked side of the magnet will be nearest the sediment. Remember to use a compass to double-check which way the north-seeking bacteria will be moving within

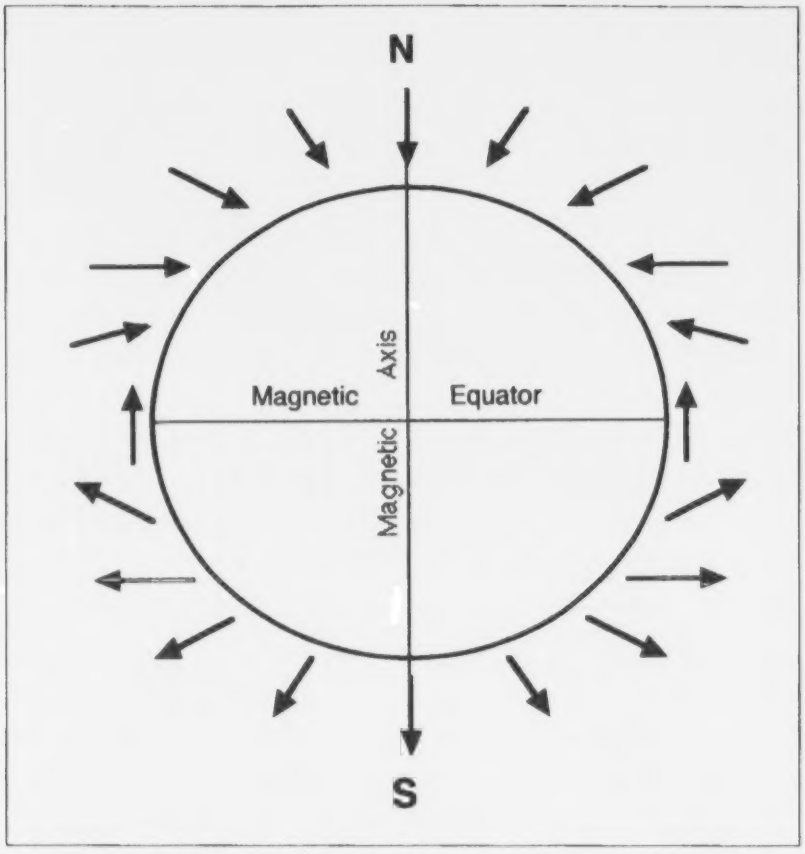

Figure 2a. The orientation of a magnetized needle (arrows) allowed to swing freely in three dimensions reveals that the Earth's magnetic force field lines are not parallel to the Earth's surface (except at the magnetic equator), but rather decline into the Earth with increasing angles at the higher latitudes.

the jar. The north-seeking bacteria will behave just like the painted end of your compass needle. Be sure that within the jar they will be swimming up towards the aerobic surface.

After you have set up the jar for the magnetic reversal, fill the jar about ⅓ full with a sediment containing a healthy population of north-seeking magnetobacteria. Cover the sediment with water from the same source. Allow the sediment to settle for a few minutes before hooking up the wire coil (or placing the magnetic lid on the jar). A great activity to test effectiveness of your reversed magnetic field is to take samples of the *surface water* before, and then again several minutes after you connect the wire terminals (or place the magnetic lid on the jar). Prior to the magnetic reversal no bacteria will be found at the surface of the water. Following magnetic reversal, large numbers of north-seeking individuals will be found at the surface.

Leave these reversed magnetic field sediments in the dark at room temperature. Students should take samples from just below the surface sediments every few days to observe changes in the population. Samples should be checked for *both* the south-seeking and north-seeking populations. Rough counts can be made by estimating the number of each type of bacteria (at opposite ends of the droplet) several minutes after exposure to the magnetic field. Generally within three or four weeks the number of south-seeking bacteria will exceed the number of north-seeking bacteria. Longer incubation times will show extreme changes in the composition of the population.

Regardless of the method used to create an environment that favors the south-seeking bacteria, it is wise to set up several jars rather than relying on one sample. It has been my experience that about one-half of the populations in the magnetically reversed samples will successfully adapt to the new environment. Extinction in a given sediment sample is a powerful opportunity for reminding the students that natural selection does not *create* polymorphisms in a population; it merely *favors* the fittest phenotypes already present. As does Fiero & Mackie's (1997) simulation on natural selection, this experiment demonstrates that "sudden, dramatic environmental changes can result in extinction if [phenotype] variability is insufficient at the time the change takes place."

Nongenetic Source of Variation in Naturally Occurring Populations

While the *production* of magnetosomes is genetically controlled, the magnetic *orientation* of the magnetosomes is *not* a function of genotype, but rather seems to be linked to the position of the line of magnetosomes during cell division. During binary fission, the cytoplasm of the cell is divided. Assuming that the line of magnetosomes transverses the equator of the cell, it is likely that each daughter cell will receive about half of the parent cell's magnetosomes (see Figure 3). As the daughter cells produce new magnetosomes to lengthen their internal "compass needles," the domain of each new magnetic particle will become aligned with the orientation of the existing magnetosomes.

Sometimes, however, when a magnetobacterium divides, the line of magnetosomes is "inherited" by only one of the daughter cells (see Figure 4). When the daughter cell lacking inherited magnetosomes begins producing its own magnetosomes, the random orientation of the magnetic particles may result in a north-seeking or a south-seeking bacterium. It is believed that such an anomaly during binary fission is the primary source of continuing dimorphism within a given population (Frankel 1996). Ordinarily, south-seeking bacteria produced in the northern hemisphere would be selected against as they move upward into highly aerobic surface waters.

Although this experiment demonstrates natural selection using a trait that does not appear to be genetically determined, the selective pressures *are* still acting on an inheritable phenotype. Using magnetobacteria for a lab testing natural selection enables your students to see that selective pressures are real and can greatly affect a living population.

Figure 2b. Most magnetobacteria found in the northern hemisphere (like this unknown cocciod species) will accumulate at the edge of a hanging droplet nearest the south end of a bar magnet (the same end sought by a compass needle). Shown at 1000X.

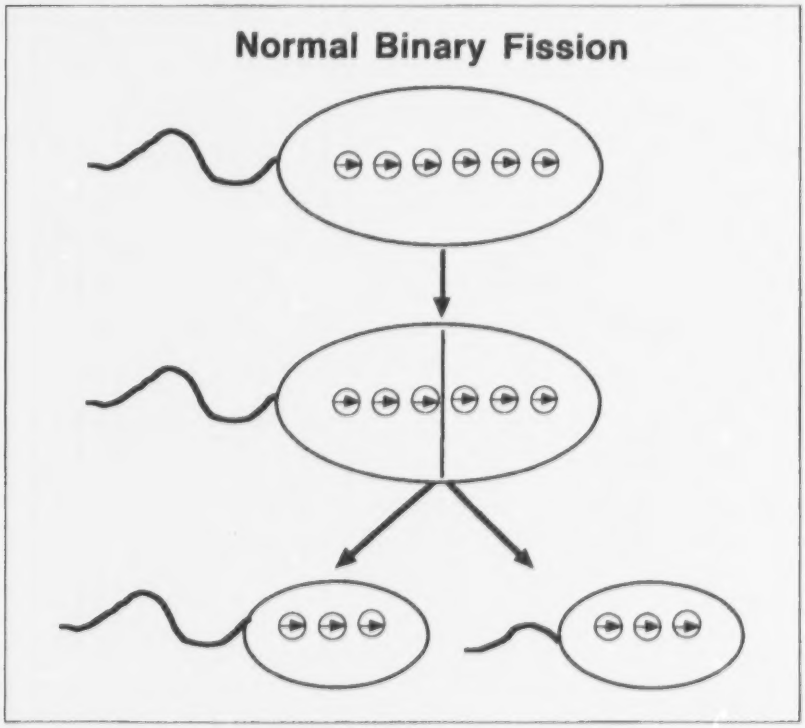

Figure 3. Normally binary fission results in both daughter cells receiving about half of the parent cell's magnetosomes. New magnetosomes produced by the daughter cells will become magnetically aligned with the inherited magnetosomes. In this way the phenotype is "inherited" although not genetically determined.

Other Ideas for Using Magnetobacteria with Your Students

1. Magnetobacteria offer a laboratory experience that teaches observational and reasoning skills. One possibility is to let your students observe the behavior of magnetobacteria BEFORE you teach about their biology. After students have observed the bacteria, use a series of guided questions to help them hypothesize and test their hypotheses. Such questions might include:
   a. What is the relationship between the magnet's position and the bacteria?
   b. Did all of the bacteria behave the same when you turned the magnet?
   c. After a few minutes of viewing, you may begin to notice that some of the magnetobacteria stop swimming. If we assume they are dead, what might be the cause?
   d. Are the dead bacteria "pulled" by the magnet? What can be inferred about the bacteria from this observation?
   e. What might be the survival advantage of this adaptation?
   f. What other organisms might be able to use the Earth's magnetic field as an aid in navigation? What advantages does such an ability bestow on an organism in terms of survival? Can you think of any disadvantages such an ability might carry with it?
2. Magnetobacteria are a great tool for teaching problem solving and complex thinking. Consider having your students compare their own swimming speed to the relative speed of the bacteria (assuming body lengths were equal). I've had my students calculate the "scaled rate" at which the bacteria swim relative to the rate at which the fastest members of our swim team move through the water. If scaled up to human size, the bacteria would be moving at about 75 to 150 meters per second! These calculations do not factor in that the bacteria are swimming with "metal weights in their pockets" (magnetosomes) and are encountering the serious resistance of Brownian motion.
3. Magnetobacteria offer a truly integrated lesson linking together life and physical sciences. They

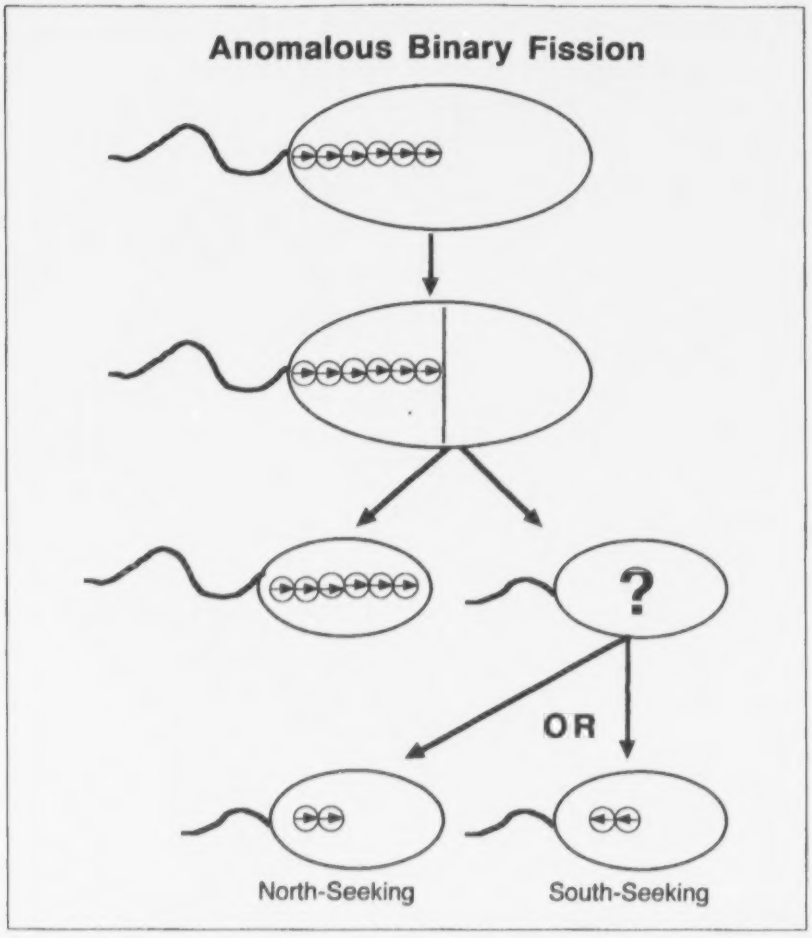

Figure 4. Occasionally binary fisson results in the production of one daughter cell without inherited magnetosomes. As this new bacterium begins producing its own magnetosomes, its magnetic orientation will be randomly determined. It may in this way become a south-seeker even though the parent cell was north-seeking.

can be used in discussions of mineralology, the Earth's magnetic field, magnetic reversals in the Earth's history, biological sources of sediment, navigational tools, evolution, microbiology, taxis, animal migrations, etc.

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Testing Folklore in the Lab

Can Common Plants Be Used To Repel Insect Pests?

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One of the best ways to teach science to students is to do labs that are intrinsically interesting to the students and that allow them to participate actively in devising and testing their own hypotheses (Gibson et al. 1999; Lung 1999). One topic that fits these criteria and that lends itself to investigation by students is the effects of plants on insect behavior. We developed a lab exercise in which students examine whether plants produce compounds that can repel certain species of insects. This lab catches students' interest in part because it addresses a topic that has been in the news in recent years: how to reduce the use of pesticides. In addition, many students who garden, or who have family members who garden, have heard folklore that suggests that some plants repel garden pests. This lab gives them an opportunity to examine this lore scientifically and thus demonstrates the scientific approach to investigating a question that is relevant for the students. We have used this lab in classes in introductory botany and zoology; however, as will be apparent, it could also be used in general biology, environmental biology, ecology, and animal behavior courses.

Background

The use and overuse of pesticides are primary contributors to water pollution and contamination of the food supply for people (Raven et al. 1998). In addition, pesticides kill large numbers of non-pest species annually in part because of the tendency for many pesticides to persist in the environment, to remain active, and to become magnified as they move up the food chain (Buchholz 1998). Because of the many problems associated with pesticides, in recent years biologists have begun looking for less toxic alternatives to extensive use of artificial pesticides. One possible alternative is the use of plant products to repel insect pests (Anonymous 1991; Buchholz 1998).

Folk wisdom has long held that certain plants, such as tansy (*Tanacetum vulgare*), French marigolds (*Tagetes patula*), and pennyroyal (*Mentha pulegium*), have the ability to repel undesirable insects, and some such plants, including the marigolds, have traditionally been used as companion plants, being planted in and around vegetable gardens specifically because people believed that such plantings would reduce damage to crops by insects.

Despite these widespread and common reports of success in repelling insects with companion planting, scientists have generally lagged behind in testing the utility of using plants to repel pests. An early exception to this rule was the development of pyrethrins, which were first made from a congener of tansy, red pyrethrum (*Tanacetum coccineum*; Brown 1995). Pyrethrins have been effective and relatively harmless pesticides. Other recent research has also in part supported the folklore; for example, scientists have found that marigolds appear capable of repelling some insect species, such as Mexican bean beetles and corn earworms, and that marigolds also repel nematodes in the soil (Gilkeson et al. 1996). Recently, there has been increasing research on natural plant products and the development of pesticides and pest repellents from these products (Winstanley 1995).

Why Should Plants Produce Compounds To Repel Insects?

Plants are a logical place to look for compounds that repel insects because of the long evolutionary history of plants during which they were developing means of defending themselves against herbivores. Because plants are unable to move away from their enemies, they have developed a wide variety of compounds that have negative effects on insects. Plants can inhibit the growth of insects that feed on them, and some plants produce insect hormones that dramatically alter the development of an insect that ingests them (Adler & Grebenok 1995). For example, bugleweed (*Ajuga remota*) produces compounds that cause insects to develop multiple head capsules as they become adults; these insects die because they cannot feed (Moore et al. 1998).

In addition to such powerful effects, plants may also exert a more subtle influence on insects: Many plant species produce compounds that can repel insect pests, either before or after the insects attempt to eat the plant (Taiz & Zeiger 1998). Currently researchers are working on identifying plants that may be pest-repellent, learning which compounds repel the pests, and genetically engineering crop plants so that these plants, too, can produce the compounds that repel pests.

The exercise below allows students to work cooperatively to determine the ability of particular plant species to repel pests. Depending on how the lab is set up, students may be allowed to choose their own plant species for testing. They could grow their own plants from seed, space permitting, or plants could be purchased from a nursery or biological supply company, depending on the species selected. Students could work together as a class, or the class may be divided into sections that cooperate in testing particular species.

Lab Preparation

A complete list of the materials used in this lab is provided in Table 1. Below we describe the species that can be used in this experiment and how to obtain them, discuss the materials needed, and explain how to set up the lab.

Insects

To use the experimental protocol described here, one should select an insect that will walk through the test container rather than fly or jump. We have used squash bugs and Colorado potato beetles as our experimental

Table 1. Materials needed to perform laboratory exercise.

Materials for Each Group of 2 Students

- 1 plastic box measuring approximately 30 X 15 X 10 cm
- 1 piece of clear, flexible plastic tubing approximately 8 to 10 cm in length and 1.0 to 1.5 cm in inside diameter
- 20 pieces of green construction paper cut to fit bottom of the box
- 20 pieces of wax paper cut to fit bottom of the box
- 20 insects
- 14.0 g of a repellent plant
- 50 ml deionized water
- Cheesecloth cut into 20 X 20-cm squares
- 2 1000-µl micropipet tips
- 2 micropipettors
- 200-ml beaker
- Data sheet for recording observations
- Pencil with eraser
- Scissors
- Mortar and pestle

General Equipment To Be Shared by Class

- Tub for soaking construction paper
- Blender
- Balance capable of weighing 14.0 g

insects because colonies of both of these species are maintained at our university. Both species are easily maintained and could be kept with little difficulty at most schools. In many locations, insects can be collected outside for much of the year, and some particularly damaging species, such as Japanese beetles (*Popillia japonica*), tend to occur in high numbers, which facilitates their collection in sufficient numbers for this exercise. Some insect species such as mealworms (*Tenebrio*) and cockroaches (*Blattella* and *Periplaneta*) can be ordered from standard biological supply companies; house crickets (*Acheta domesticus*) are generally available more cheaply from pet stores and bait shops.

Pest-Repellent Plants

Growing plants from seed gives students the most choice in selecting plant species to test. At our university we have grown plants from seed in a greenhouse for this exercise. Plants may also be grown in a classroom if there is sufficient sunlight or artificial light from grow-lights. If light or space is inadequate, plants may also be purchased from local nurseries, mail-order plant catalogs, or biological supply companies; we have used marigolds purchased from a local nursery and obtained good results. Species we have tested that have repelled both squash bugs and Colorado potato beetles include French marigolds, stinking Roger (*Tagetes minuta*), tansy, English pennyroyal, mugwort (*Artemisia vulgaris*), and wormwood (*A. absinthium*). Many other species have been suggested as pest repellents; popular gardening guides sometimes list such plants (see for example Riotte 1975; Denckla 1993; Brown 1995), and students may have their own suggestions based on their experiences.

Controls

Two different controls may be run for this experiment, one comparing the reactions of insects to an extract of a potentially pest-repellent plant and to water, and one comparing their reactions to extracts from a pest-repellent plant and from a non-repellent plant. For a non-repellent control plant, one should select species that are not producing large amounts of aromatic compounds and that are neutral to the insects being tested (i.e. one would not choose to use a squash extract as a control if squash bugs were being tested). We have often chosen to use species that provide food for humans, as many of these species seem to produce lower levels of aromatics than do many wild species. Species that we have used with success include carrots and spinach; leaf lettuce and other similarly mild-flavored species might also be suitable. As with pest-repellent plants, control plants may be grown from seed or purchased, although purchasing suitable plants for controls may be more difficult during the winter season.

Containers for Choice Tests

The plastic shoeboxes should be modified for insect choice tests as follows (see Figure 1A). Cut a round hole in the center of one of the long sides of each box; this hole should be located near the bottom of the box and it should be sized so that the plastic tubing will fit snugly through it. Insert a piece of plastic tubing approximately 8 to 10 cm in length into the hole; when inserted, the tube should rest on the bottom of the box and extend just far enough into the box to be stable (approximately 1 to 2 cm). The plastic tube serves as the entryway into the container for the insects being tested and helps to ensure that the insects do not turn around and leave the box, and that they are facing directly forward when they enter the box. Thus, it is helpful if the tube diameter is not substantially larger than the width of the insect being tested. The ideal diameters of the hole and the tubing may accordingly vary somewhat depending on what species of insect is being tested; the measurements given have worked for both Colorado potato beetles (*Leptinotarsa decemlineata*) and squash bugs (*Anasa tristis*), which are approximately 0.5 to 1 cm in width, including their legs.

Before running a trial in the shoebox, one piece of construction paper and one piece of wax paper should be placed in the box with the wax paper on bottom (see **Running the Trials** below). The wax paper is used to prevent the extract from soaking the bottom of the box in which the trials are run, which would make clean-up between trials difficult. The green construction paper is needed because on lighter paper the plant extract shows up as a dark green band, whereas the water is clear. We found in early trials of this system that some insects were attracted to the dark color of the extract, which confounded the results.

About 15 minutes before the lab, the pieces of construction paper to be used should be set to soak in deionized water. This soaking ensures that the paper will absorb the extract well. About 5 minutes before running a trial, pieces to be used should be removed from the soaking water and set out on a table or rack so that they drain slightly; they should be thoroughly wet, but not dripping when used.

Preparing the Extracts

As the extracts lose their efficacy relatively quickly over time, they should be prepared during the lab exercise. This can be done by the students. To make an extract, follow the steps given below.

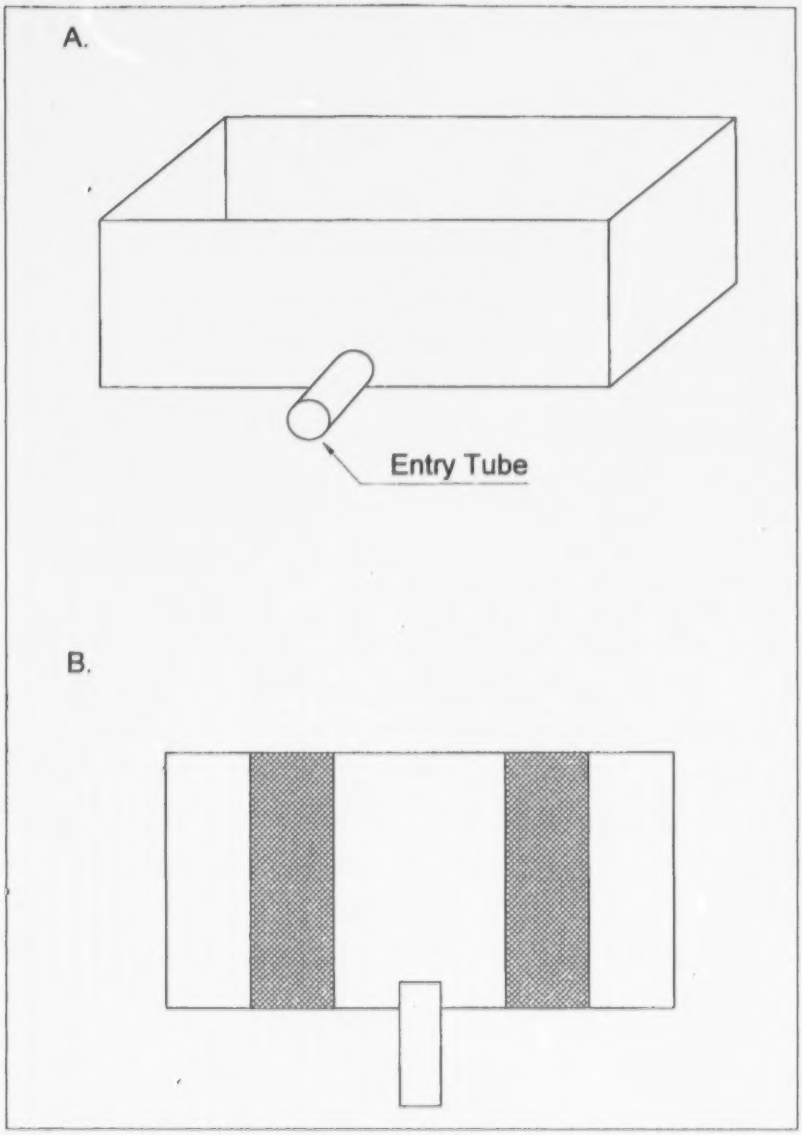

Figure 1. A. Side view of box used for insect trials, showing the placement of the plastic tubing through which the insect being tested enters the box.

B. Top view of box ready for a trial. The cross-hatched areas represent the locations of the two substances being compared; typically, a repellent plant extract would go on one side and a control extract or water on the other.

Step 1. Use scissors to cut up the plants to be tested into pieces small enough to fit easily into a weighing pan. All the above-ground parts of the plant can be used, including stalks, leaves, flowers and buds, although if the stalks are very thick they will be difficult to grind up.

Step 2. Weigh out 14 g of the plant per student group.

Step 3. Each 14 g of plant material should be chopped up as finely as possible with scissors, and then placed in the mortar with 50 ml deionized water and ground up with the pestle. With some plants it works best to add all the water at once; with others, adding the water gradually seems to make the grinding easier. Grind the mixture well with the pestle.

Step 4. After grinding the mixture, place it into the blender and puree it. The mixture is ready when it is liquefied enough that it stays in the bottom of the blender during pureeing.

Step 5. Now filter the mixture through the cheesecloth. To remove the solids from the liquid extract, a student should be wearing gloves so that the extract is not contaminated. For convenience, place the cheesecloth square on top of the mortar, pour the mixture from the blender onto the cheesecloth, then gather up the cheesecloth around the mixture and squeeze the liquid out and into the mortar. This liquid is the extract that will be used in the trials.

Running the Trials

Step 1. In the bottom of the container, place a piece of wax paper and then a piece of damp, green construction paper. Both papers should be placed under the edge of the entrance tube so that an insect coming into the container will step out onto construction paper.

Step 2. The substances to be compared should now be added to the container, one on each side of the entrance tube (see Figure 1B). For a trial comparing a pest-repellent plant extract to water, for example, use a micropipettor to put 1,000 µl of the extract in a band 5-cm wide running across the container. This band should begin approximately 4 cm to one side of the entrance tube. Then, using the other micropipettor, put 1,000 µl of deionized water in a band 5-cm wide, beginning at the same distance from the tube on the opposite side. The liquids should not be allowed to run outside the bands; they should not do so if they are applied to the paper slowly.

Step 3. Immediately after adding the substances to be compared, place an insect in the entrance tube. Most individuals will walk through the tube and into the box, either immediately or after some seconds. If the individual does not move forward, it may be coaxed to do so by prodding it gently from behind with the eraser end of a pencil. The individual being tested should enter the container walking straight ahead and on the bottom of the tube. Students may need to rotate the entrance tube to ensure that the insect enters the container properly.

Step 4. Note the behavior of the insect after it enters the container, and record its first reaction to one of the substances being tested. The possible reactions include positive and negative reactions to whichever substance it first encounters. A positive reaction consists of moving toward a substance

and then crossing the area on which that substance is laid down. For example, if a squash bug enters the container, goes toward the water and then walks over the part of the paper on which the water was added, that individual is recorded as having a positive response to water. A negative reaction consists of moving toward a substance but then stopping before crossing it. Insects that have a negative reaction generally then turn and move toward the other substance or attempt to get out of the container, either by going back through the entrance tube or by climbing the back wall of the container. Depending on the level of students doing this lab, instructors may want to alert them to more subtle cues: insects can also display a negative reaction by stopping and rearing back, or sometimes by speeding up suddenly when crossing a substance. Even without adding these behaviors, which may be difficult for younger biologists, the results should be clear.

In order to record these reactions, students can use a data sheet divided into four columns that would include positive and negative response columns for each substance; then for each trial students would need only to place a tally mark in the appropriate column. In some cases an individual may have more than one response, e.g. an individual could approach the extract and turn back (a negative response to the extract) and then approach the water and also turn away (a negative response to the water). To simplify data recording and analysis, only the first response of the insect should be recorded, and this should be made clear to students. Occasional individuals will not appear to make a choice but will instead either march directly to the back of the container and attempt to climb the side, or turn around and attempt to exit through the entrance tube. These individuals can be recorded as having no response and eliminated from consideration in analyzing the results.

Step 5. After each trial is run, the used pieces of construction and wax paper should be removed from the container and thrown away. The container should be wiped out to be certain that none of the extract remains on it, and new paper can then be added to the container to ready it for the next trial.

Step 6. Run additional trials. In subsequent trials, the students should alternate the positions of the two substances being compared, i.e. if in the first trial the plant extract is on the left in the container and water is on the right, then these positions should be reversed in the second trial. This method ensures that the insects aren't responding to some factor other than the extract, such as a source of light or heat in the room.

Organizationally it is generally simpler for each pair of students to focus on making one comparison, e.g. running all trials with a particular extract vs. water or with two particular extracts, one from a potentially pest-repellent plant and one from a control. Depending on the size of the class, half the class might test an extract vs. water and the other half the same extract vs. a control plant. These two tests combined should allow students to determine the efficacy of their chosen plant as a repellent.

Data Presentation & Analysis

For lower-level biology classes, discussing or graphing the results of the experiment may be sufficient. For more advanced students, statistical analysis of the data obtained would be desirable.

Within a class, the data from all student groups that are testing the same hypothesis should be combined and the totals analyzed. The simplest way to analyze the data is to divide the responses into those that moved away from the pest-repellent extract and those that did not. In other words, total the number of individuals that had a positive reaction to the water or a negative reaction to the extract; this can be considered as the number that avoided the extract. The remaining individuals, which had either a negative response to water or a positive response to the extract, are the ones that did not avoid the extract.

By chance, one would expect 50% of all individuals to move toward the extract and 50% to move away. To determine whether the results students obtain are statistically significant, run an X^2 test that compares the values actually obtained by students with the expected values.

Students may also be encouraged to write up their results, either in a scientific format or in a format accessible to the public. This is a topic that is approachable for non-scientists, and students' parents and the general public are likely to be interested in the results of these experiments.

Using & Extending This System

The experiment as described above will test the hypothesis that an extract made from a certain plant will repel a particular species of insect. Other related questions may also be asked using this same system, and students may come up with some of these questions after obtaining their results from the experiment described above. For example, if both leaves and flowers are used in making the extracts, students may wonder whether only one part of the plant is causing the observed effects; this can be determined by testing leaves and flowers separately.

This lab can be modified depending on the level of the students and on the level of active learning desired. First, it can be completely planned by the instructor, and the plants to be tested and experimental methods given to the students. Alternatively, the first trials could be planned, with students testing, for example, water versus a repellent plant extract, after that each group of students could devise its own test. For the highest level of student participation, the instructor might introduce the topic and tell the students what materials are available; then the students would be responsible for designing the tests, either in small groups or as a class.

A question that our students have asked is what effect changing the concentration of the extract would have on the insects' behavior. Answering this question may be more suitable for university-level courses than for students at earlier stages. This may be addressed by performing a series of dilutions of prepared extract and performing choice tests with those dilutions; we have found that the behavior of the insects is generally random (i.e. 50% move toward the extract and 50% move away) at a one-eighth dilution; for some species of plants, the effects on insects' behavior are lost when the extract is more concentrated. If the class works together and tests the effects of different dilutions of the extract, the class can construct a dilution curve that will show that the effects on insects decrease with decreasing concentration.

Conclusions

In this laboratory, students get hands-on experience at several different aspects of the scientific method, including devising a hypothesis, generating predictions from and testing that hypothesis, collecting and summarizing data, doing statistics, and presenting their results. The results from the experiments could have practical applications, either for gardeners or, in the future, for farmers attempting to reduce the use of pesticides—and

the practical aspect of this experiment has appealed to our students. After doing the lab exercise, students can tell their acquaintances whether, for example, planting marigolds (which are widely reputed to repel a variety of pests) around their tomatoes might reduce pests, and the instructor can encourage the students to think about applications by asking them to make recommendations to gardeners based on their findings in the lab. The combination of doing the lab experiment and thinking about potential applications helps students to see the links between the way science is done and the potential of some research to affect their lives by improving the quality and health of Earth's environments.

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The Winogradsky Column & Biofilms

Models for Teaching Nutrient Cycling & Succession in an Ecosystem

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Wetlands are among the most productive ecosystems in the world. The high productivity of wetlands is due to their ability to capture large amounts of the sun's energy and store it as chemical energy, as well as to their efficient recycling of what is produced (Niering 1985).

The characteristic hydric soils in wetlands reflect the effect of water when present on soil or substrate for extended periods. According to Mitsch and Gosselink (1993) hydric soils that are semipermanently or permanently flooded develop gray or sometimes greenish or blue-gray color as a result of a process known as gleying. This process is the result of the chemical reduction of iron. Another characteristic of soils that are seasonally flooded is mottle formation. Orange/reddish or dark reddish-brown/black mottled spots seen throughout an otherwise gray soil matrix suggest soils with spots of iron and manganese oxides in an otherwise reduced environment. The development of gleys and mottles is mediated by microbiological processes and the rate at which they are formed depends on the presence of sustained anaerobic conditions, appropriate soil temperature (5° C is considered optimum), and the presence of organic matter as a substrate for microbial activity. These chemical transformations in wetland soils illustrate the interaction of nutrients and microbiological activities in ecosystems.

One problem faced by biology teachers in teaching nutrient cycling is the lack of suitable exercises that can be conducted in the classroom. We feel that the use of a Winogradsky column and biofilms can help junior high and high school students develop a clearer understanding of these chemical transformations by presenting a vertical model and the microorganisms participating in the process of succession.

The Winogradsky column, named for the Russian microbiologist, Sergei Winogradsky, is a miniature ecosystem that illustrates microbial succession of several groups of microbes. Winogradsky devised the column in the 1880s to study soil microorganisms (Madigan et al. 1997). The column is prepared by enriching a sediment sample (soil or mud) from a lake, pond, stream or seashore with a nutrient substrate and allowing it to incubate at room temperature for several weeks. To study microbial succession, several glass microscope slides are partially inserted, vertically, into the top of the soil or mud sample. In time, the glass microscope slides serve as a surface to which bacteria, and later algae, protozoans and invertebrates attach and form biofilms. Observing these biofilms over several intervals of time illustrates microbial succession.

We used this laboratory activity with 20 junior high science and high school biology teachers at a summer workshop on wetland ecology. During the following academic year, the teachers constructed Winogradsky columns and studied biofilms at their respective schools. At the annual evaluation of the project, teachers reported on how they involved their students in investigating the changes that occurred as their Winogradsky columns developed. The exercise was conducted using an inquiry-based approach with students working in cooperative learning groups. Based on earlier activities involving the characteristics of wetland soil, e.g. color, odor and texture, and microbe habitats (Kesselheim & Slattery 1995), students were asked to predict what might happen to the column sediments after several weeks. Nutrient cycling and biological succession are generally presented in textbooks as abstract theoretical concepts that students often find "difficult" and "boring." This exercise helps students to conceptualize and interpret the changes going on under aerobic, as well as under low oxygen conditions, and to identify chemical changes that might affect the activities of the microorganisms in the soil. Changing areas of pigmentation and the kinds of microbes that appear and disappear with changing oxygen and hydrogen sulfide levels are recorded in the students' laboratory journals.

The objectives of this exercise include: setting up a Winogradsky column and biofilm slides, interpreting the chemical transformations that occur in the column as a result of color changes in the soil, identifying common microorganisms, and determining the microbial composition of the column over specified intervals of time.

Materials

- One clear or transparent (plastic or glass) container with straight sides of about one liter capacity, with a diameter of at least 5 cm and a height of at least 17 cm (We used a tall, cylindrically shaped, empty plastic water bottle cut off below the neck.)
- Wetland soil or mud from a lake, pond, stream or seashore, enough to fill the container about ⅔ full
- Plastic wrap or aluminum foil to serve as a lid for the column
- Scoop for handling mud or soil
- 5 g $CaCO_3$ (calcium carbonate)
- 5 g $CaSO_4$ (calcium sulfate)
- 10 g finely shredded newspaper or other cellulose source such as sawdust, leaves, shredded woodchips, grass clippings
- Balance to weigh the chemicals
- 8 to 10 glass microscope slides per column for the biofilms
- Permanent marker to label slides
- Coverslips
- (Optional) A simple stain e.g. methylene blue stain (Morholt & Brandwein 1986)
- (Optional) Gram staining reagents: crystal violet, Gram's iodine, ethyl alcohol, safranin (Morholt & Brandwein 1986)
- Slide holder or forceps (We used clothespins as holders.)
- Squeeze bottle containing water
- Bunsen burner
- Microscope

Procedure

A. Construction of the Winogradsky Column

(modified from *Microbiology Techniques* by Kelly and Post 1991)

This laboratory activity should be planned on a long-term basis of about two to three months (or longer) for optimum column development.

1. Estimate an amount of soil or mud to fill the container ⅔ full. Remove any rocks, twigs, or any objects. Mix the soil or mud with 5 g $CaSO_4$, 5 g $CaCO_3$ and 10 g of a cellulose source (shredded newspaper, grass clippings, leaves, etc.). The cellulose serves as the source of organic carbon.
2. Place soil or mud in the container and add sufficient water to cover the soil 3 to 4-cm deep. Stir the mixture to eliminate air bubbles.
3. Place 8 to 10 clean glass microscope slides in a vertical position on top of the soil, leaning them against the side of the container. At least 70% of the length of the slide should be submerged in the water.
4. Mark the water level of the column and cover the top with plastic wrap or aluminum foil to reduce evaporation. You may need to replace water, to the original level, over a period of time.
5. Incubate the column at room temperature for several weeks in a north window, if possible, so as to receive adequate, but not excessive, sunlight.

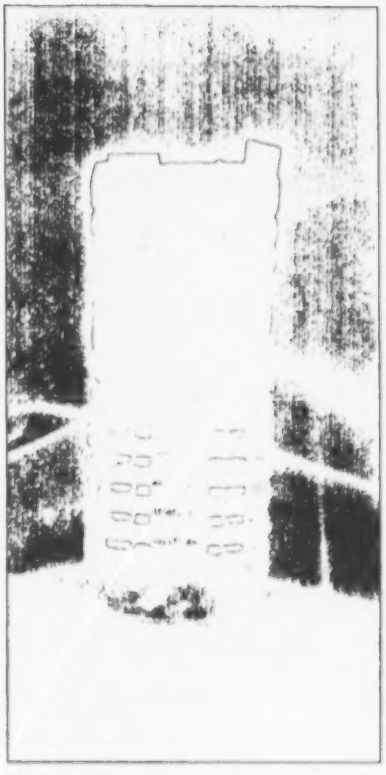

Figure 1(a). Placement of glass slides in the Winogradsky column.

Figure 1(b). Cover added to reduce evaporation from the column.

B. Observations of the Winogradsky Column

Students are encouraged to make daily observations during the first week of the setup. Subsequently, weekly observations are sufficient.

1. Note the appearance of the column and draw any changes in color patterns or growth that occur in the soil and water over the incubation period. Some bacteria will "bloom" and then disappear as others colonize. Record any differences that appear in the column and compare columns among students.
2. Remove the foil cover. Describe and record any changes in odors in the column. Have students compare odors of their column.
3. Students are asked to visualize the oxygen and hydrogen sulfide gradients that are formed in the column, going from top to bottom, and from bottom to top, respectively, and to diagram the changes that occur.

C. Observing the Biofilms

1. Slides should be removed on Day 2 to observe the formation of the biofilm and the succession that is taking place. Because organisms will colonize both sides of the glass slides, one side should be wiped clean.
2. Examine slides daily for at least 5 days, then at weekly intervals for several weeks (or even months), using a wet mount, under low and high power magnifications (Morholt & Brandwein 1986). Once the observations have been made, the slides may be marked with the observation date and returned to the column to be observed again at a later date. Over time, microbes will continue to adhere to the glass slides. Additional slides may be added to the column to "restock" the supply. Notations written with a permanent marker may be used to mark the date as slides are added to the column.
3. As an optional activity, slides may be stained with simple and/or differential stains (such as the Gram stain) and examined under high power and oil immersion lenses. These stained slides should not be returned to the column.
4. Note the changes that take place over a period of time with respect to the type and abundance of macro and microorganisms. You may need to modify your fixing and staining methods depending on how long the slides have been immersed in the column. For example, during the first days of observation, Gram staining may be more successful as more bacteria colonize the slide. As time passes and the biofilm develops (and more microbes encrust on the slide), a wet mount, with or without the simple stain, would be more effective for viewing the larger eukaryotes. Bacteria are usually the first to colonize, followed by an assortment of algae, protozoans and invertebrates.

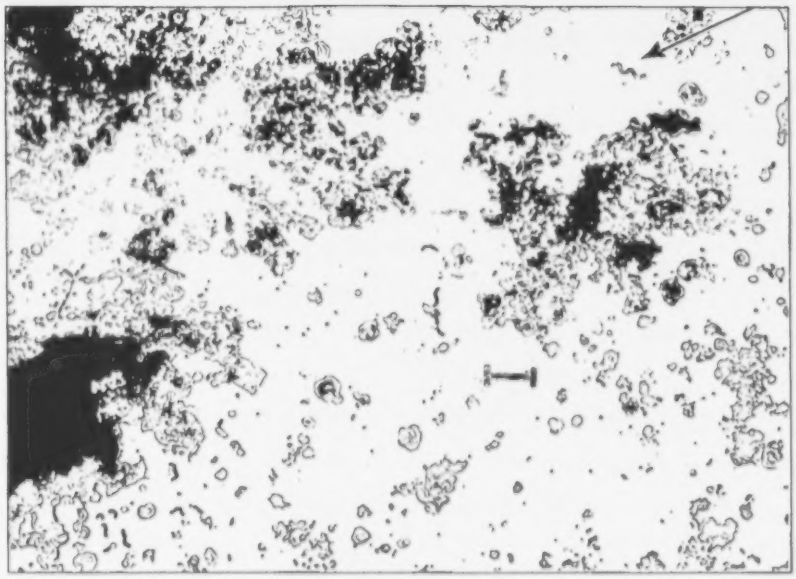

Figure 2(a). Biofilm development over three weeks.
Week 1: Several spiral-shaped bacteria (10μm = bar) are seen.

Figure 2 illustrates biofilm development over a period of three weeks.

Discussion

The Winogradsky Column

The Winogradsky column is a miniature ecosystem in which microorganisms and nutrients interact over time. As oxygen diffuses downward from the surface, fermentation products from the breakdown of cellulose and hydrogen sulfide diffuse upward from the reduced lower zone (Prescott et al. 1996).

As microbial metabolites migrate within the column in response to various chemical gradients, groups of organisms accumulate in accordance with their required conditions in much the same way that they do in nature, thereby simulating the vertical microbial distribution that may be found in lakes, ponds, or in the intertidal zone. Figure 3 is a diagram of the Winogradsky Column (modified from Prescott et al. 1996).

In the lower region, anaerobes such as *Clostridium* degrade cellulose and produce fermentation products such as organic acids, alcohols and hydrogen.

These fermentation products together with sulfate (from the calcium sulfate enrichment) are used by sulfate-reducing organisms such as *Desulfovibrio* to produce hydrogen sulfide. The development of sulfate-reducing bacteria appear as blackened areas in the lower portion of the column and may even blacken zones throughout the sediment if sufficient aerobic bacteria are present to deplete the oxygen supply.

Figure 2(b). Week 2: Protozoa (20μm = bar) colonize the biofilm.

The sulfides are then used by anaerobic photosynthetic populations such as green sulfur, *Chlorobium*, and purple sulfur, *Chromatium* bacteria. Evidence of this is seen as purple and green patches in areas throughout the column as these phototrophs respond to gradients of light and sulfide. In nature, purple and green sulfur bacteria may be found in any fresh or marine waters as long as there is a sufficient supply of hydrogen sulfide and the water is clear enough so that light penetrates to the anoxic (anaerobic) zone (Madigan et al. 1997).

Ferric compounds may precipitate and appear as brown or greenish-gray deposits. The predominant iron-reducing bacteria within gleyed soils appear to be *Bacillus* and *Pseudomonas* (Atlas & Bartha 1993.)

Higher up in the column, in the microaerophilic zone, rust-colored patches will appear, generally from the photoheterotrophs, such as the purple nonsulfur bacteria (*Rhodospirillum* and *Rhodopseudomonas*). These organisms trap light energy and use organic molecules as both electron and carbon sources.

At the mud-water interface, where both hydrogen sulfide and oxygen are found, various aerobic, sulfide-oxidizing organisms such as *Beggiatoa*, *Thiothrix* and *Thiobacillus* may colonize. This water is frequently quite turbid.

Algae and cyanobacteria appear quickly in the upper portion of the water column, where sunlight is abundant. By producing oxygen, these organisms help to keep this zone aerobic. This watery top layer contains an interesting diversity of microbes—green algae, cyanobacteria, various aerobic bacteria, fungi and protozoa.

Nutrient Cycling

Nutrient cycling of elements between the atmosphere, water and soil results in the biogeochemical zonation of habitats that can be seen in the column. Although the column is not connected to the environment, cycling of elements does occur as macro and microorganisms degrade various organic substances found in the soil/mud and in the "enrichment materials" that have been added. While the column mimics the natural wetland in several ways, the dynamic interplay found in the natural environment is missing. For example, additional elements are not being continuously added to the system as they are in nature, nor are microbes being "transported" into the system by runoff, wind or other forces.

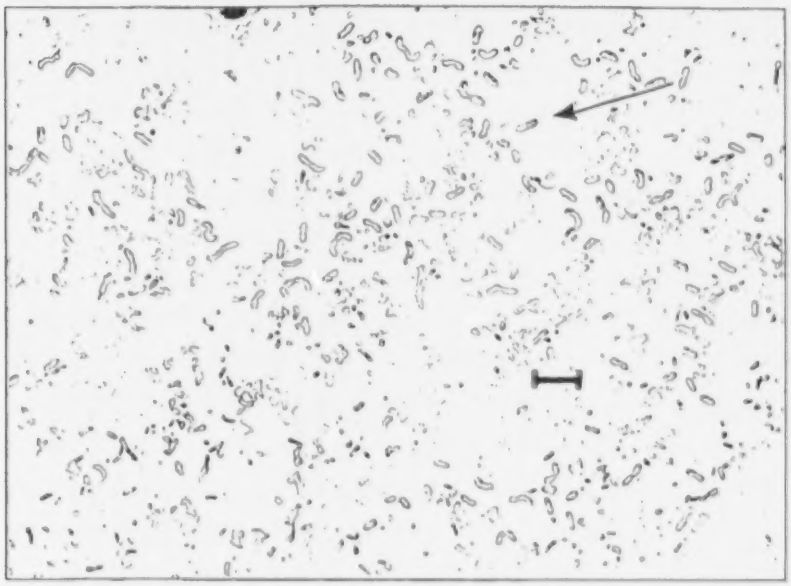

Figure 2(c). Week 3: Numerous rod-shaped bacteria (10μm = bar) are noted.

In the column, carbon, hydrogen and oxygen are cycled primarily by photosynthesis and respiration. Under anoxic conditions, organic carbon may be recycled in the fermentation process. As alcohols and acids accumulate, they may be metabolized further with the simultaneous reduction of nitrate or sulfate.

Changes in the oxidation states of sulfur are mediated by microorganisms, establishing the sulfur cycle (Atlas & Bartha 1993). For example, algae and many heterotrophic microorganisms may utilize sulfate, incorporating it into protein. Other organisms may reduce this sulfate to produce hydrogen sulfide. Hydrogen sulfide may then be subject to microbial oxidation under aerobic conditions, or may be used by the anaerobic photosynthetic sulfur bacteria (Atlas & Bartha 1993). Hydrogen sulfide gas may diffuse through the column, as it does within natural sediments, resulting in the characteristic odor associated with anoxic soils. As part of this exercise, students are asked to describe the odors created within the column.

In the nitrogen cycle, nitrogen from the atmosphere moves through various organisms and back into the atmosphere. The critical steps of nitrogen fixation, nitrification, and denitrification are all mediated by bacteria and are responsible for this cyclic flow (Atlas & Bartha 1993).

The cycling of iron involves the oxidation-reduction reactions that reduce ferric iron to ferrous iron and oxidize ferrous iron to ferric iron. Ferric iron reduction is a common occurrence in soils, bogs and anoxic lake sediments (Madigan et al. 1997).

Although students are not asked to identify the microorganisms in the sediments according to specific genera, they are able to observe visual changes that correspond to the diversity in the column. They are able to microscopically identify groups of microbes (such as bacteria, algae, fungi and protozoa) from the water column using their textbook and other laboratory resource materials.

Biofilms

Biofilms develop on surfaces immersed in natural, aqueous environments. These surfaces may be aquatic plants and animals or submerged objects such as wood, plastic, metal, concrete or rocks. Biofilms form rapidly in fluid environments and increase over time, especially where there is nutrient accumulation (Madigan et al. 1997).

The development of biofilms is initiated by microorganisms forming a monolayer attachment on the surface. Over time, this film becomes more complex with layers of organisms of different types colonizing the surface. Depending on the energy sources available, photosynthetic microbes, facultative chemo-organotrophs, or sulfate-reducing microorganisms may be present (Prescott et al. 1996).

The formation of biofilms has broad implications not only in natural hydric systems, but also in industrial microbiology and in public health. Heated water supplies and potable water systems may form slimy aggregates. In air-conditioning and other water system units, biofilms may serve to protect pathogenic bacteria from the effects of chlorination and other antimicrobial agents. Biofilms may also colonize the surfaces of medical equipment such as urinary or intravascular catheters and dialysis units and may serve as reservoirs of infection. In our own bodies, biofilms form plaque that leads to tooth decay. Biofilms can also accumulate on contact lenses, where bacteria may cause eye irritations and infections (Prescott et al. 1996).

Classroom Applications

Each Winogradsky column is unique and serves as an ideal experiment for inquiry-based laboratory activities. Students can be imaginative and collect water from neighborhood habitats—ponds, lakes, streams, puddles, birdbaths, or tidal creeks; use different soil types; and supplement the soil with various cellulose sources that may include shredded newspaper, grass clippings, sawdust or wood clippings. The variety of organisms and pigmentation in the column varies depending upon the nature of the soil and the nutrients contained in the sediments. Hence, there is no predetermined result in this experiment.

In this study, we collected feedback from the teachers about their students' reactions to the Winogradsky column and the biofilm experiments. We also collected the students' laboratory journals containing experimental data and perceptions. In addition, the second author observed classes where the experiments were conducted. Summarizing from various sources of students' comments, the Winogradsky column and biofilms exercise is a positive experience in light of the following instructional merits:

1. The exercise enhances the understanding of scientific principles and processes. The Winogradsky column serves as a source of many different types of microbes involved in nutrient cycling. Many of these organisms play an essential role in the transformations of carbon, nitrogen, sulfur and iron. Information-processing skills are developed as students predict and explain their results.
2. Cooperative learning groups were used to organize and manage the

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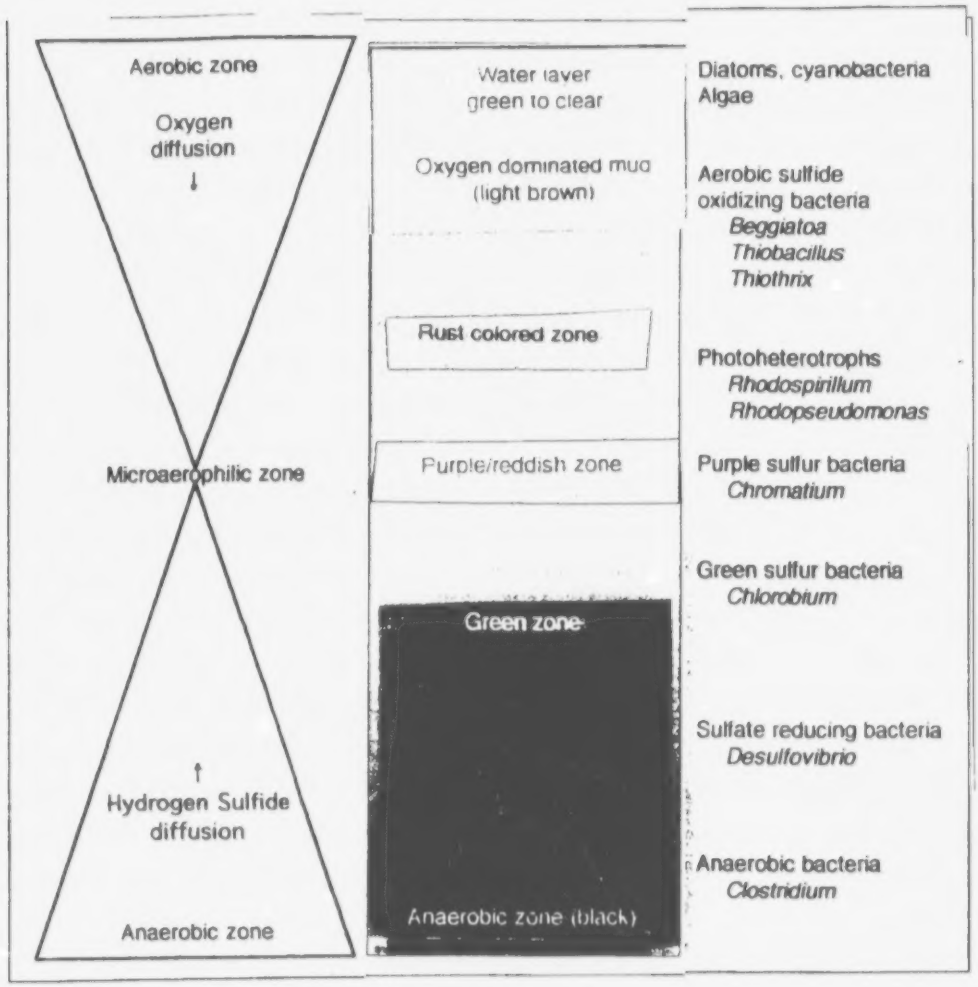

Figure 3. The Winogradsky Column (modified from Prescott et al. 1996).

lesson's activities. This classroom management method promoted peer relationships and created positive interdependence among students to achieve the lesson outcome. The predictions, summarized as the group's prediction, demonstrated the students' ability to synthesize knowledge and experiences about the characteristics of wetland soil (i.e. color, texture and odor) from earlier lessons. The knowledge gained from the Winogradsky and biofilms activity was not acquired from rote memorization but from conceptual learning that required the use of prior activities and the accommodation of the new information into the existing mental structure about wetlands.

3. The Winogradsky column with biofilms is an investigative activity that may be used over an extended period of time. Students learn and practice multiple science process skills such as observing, predicting, inferring, generalizing and interpreting data. The Winogradsky laboratory activity provides students with manipulative practice in setting up the column and with the intellectual challenge in finding out if their predictions are observed in the developed column. The relevance of this laboratory activity is maintained over a long period as students rely on these established procedures of inquiry to find the best explanation for their observations. In addition, this exercise integrates earth science and geochemical cycles in the life science concept of interdependence of organisms.
4. The changes in the Winogradsky column, such as the appearance of color and mottle formations, are models and visual anchors for identifying anaerobic and aerobic processes and for describing the role of microorganisms in nutrient cycling. Based on what they observe, students develop an understanding of decomposition and chemical transformations that serves to augment what they read from the textbook or hear in a lecture.
5. The students' observations of microorganisms that colonize the biofilms reinforce and expand the concept of ecological succession. As one student wrote in her journal, "It is like a parade of microbes—they follow one another and they multiply until there are many of different kinds."

Nutrient cycling is one of the concepts in the unifying principle of *Interdependence of Organisms* included in the Content Standard for Life Science in grades 9 through 12. Teaching

Standard A suggests that "teachers of science plan an inquiry-based science program for their students" (*National Science Education Standards 1996*). Nutrient cycling is an abstract concept to most junior high and high school students, many of whom may not yet have reached the level of formal reasoning. The observations that are made as the column develops provide students, who may be in transition from concrete to formal reasoning, with an observable model of nutrient cycling and microbiological succession. During the long-term observations, students visualize the gradual changes that take place during the chemical transformations and identify the role of various microbes involved in the process.

Our study illustrates that the Winogradsky column and biofilms is a simple, inexpensive and safe exercise that junior high and high school students can perform using sediments from their local environment. Active participation by the students adds to the interest and relevance of the experiment and integrates critical thinking with an investigative approach. Personal involvement increases excitement and discussion and provides an opportunity for students to experience how scientists function in the real world.

Acknowledgments

The authors wish to thank the 20 teachers who participated in the 1994 and 1996 Freshwater and Wetland Ecology Summer Institutes for their cooperation during the classroom observations and for providing us with the student journals and evaluation reports on the Winogradsky exercise. Most of all, the authors commend the teachers' dedication and commitment to teaching biology as a process of knowing.

Many thanks to Dr. Kenneth Curry, a colleague in the Department of Biological Sciences, for producing the photographs for this article.

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Appendix A

Procedures for preparing the wet mount, simple stain, and Gram stain

(Morholt & Brandwein 1986)

Wet Mount Technique

1. Place the slide containing the biofilm on a flat surface. Since the biofilm accumulates on both sides of the slide, one side should be wiped clean.
2. Handle the coverslip carefully and place the edge of the coverslip on the slide so that it touches the edge of the water.
3. Rapidly lower the coverslip onto the slide to prevent forming and trapping air bubbles.
4. Place the slide on the microscope stage and observe under low power. Note the size, shape and arrangement of microorganisms. Record your observations by drawing and writing a brief description.
5. As an optional activity, examine the slide under high dry and oil immersion. Some microbes are motile, while others exhibit Brownian movement. Record your observations.

Appendix B

Simple Staining Technique

1. Allow the slides to air-dry on a flat tabletop.
2. Heat-fix the organisms. To heat-fix the smear, hold the slide with a slide holder and pass the slide back and forth three times immediately over the flame of a Bunsen burner. Heat-fixing kills bacteria and they adhere to the slide.
3. Flood the slide with methylene blue. Allow the stain to react with the organisms on the slide for 1 minute.
4. Using a slide forceps, tilt the slide so the stain runs off into a staining pan or sink.
5. Using water in a squeeze bottle, wash off the excess stain as the slide is tilted over the pan or sink.
6. Blot the slide gently with bibulous paper or a paper towel to remove the water. Do not rub the top part of the slide because that will remove the stain.
7. Place the slide on the microscope stage and observe under the low power.
8. Note the size, shape and arrangement of microorganisms. Record your observations by drawing and writing a brief description.
9. As an optional activity, repeat using high dry (40X) and oil immersion (100X) lenses.

Appendix C

Gram Staining Technique

1. Allow the slides to air-dry on a flat tabletop.
2. Heat-fix the smears.
3. Flood the slide with crystal violet, and allow it to react for 1 minute. All the bacteria will stain blue/purple.
4. Hold the slide with a slide handle or forceps, tilt it and drain the dye off into the staining pan or sink.
5. Using the squeeze water bottle, rinse the slide with a gentle stream of water.
6. Flood the slide with iodine. Allow the iodine to react for 1 minute.
7. With a slide forceps, tilt the slide and allow it to drain.
8. Immediately rinse the slide with water from your wash bottle.
9. Decolorize the slide for about 30 seconds by allowing the alcohol to run over and off the smear. The Gram positive bacteria retain the blue/purple color; the Gram negative bacteria lose their stain and become colorless.
10. Rinse immediately with water from the wash bottle to stop the decolorizing action.
11. Flood the stain with safranin counterstain for 1 minute. Drain the slide.
12. Rinse the slide thoroughly with water from the wash bottle. The Gram positive bacteria retain the blue/purple color; the Gram negative bacteria stain a pink/red color.
13. Blot the stained slide in a booklet of bibulous paper or between layers of paper towels. Do not rub, because that will remove the smear.
14. Place the slide under the microscope. Place a drop of oil immersion on the slide and observe under the oil immersion lens (100X).
15. Identify the bacteria as Gram positive or Gram negative. Record your observations by drawing and writing a brief description.

Using Cemetery Data To Teach Population Biology & Local History

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The problems posed by modern society can no longer be addressed by individuals who are narrowly trained in the traditional disciplines. If life on our overcrowded planet is to be rewarding for the individual, citizens of the 21st Century must be scientifically literate, culturally integrated, politically astute, and able to communicate with others. Many of our students are rightfully demanding to know how each subject they take is relevant to their lives and to their preparation for the future. Science teachers have a responsibility to help students learn not only the principles of science, but to relate them to the community in which they live.

The activity described here involves the collection and analysis of biological data, research into the social and medical history of the community, and the development of skills in oral and written communication. It is inexpensive, can involve an entire class, and can be designed as an interdisciplinary, cooperative study among teachers of biology, math, social studies and English.

Background

A population is an assemblage of individuals of the same species inhabiting a given area (Krebs 1994). Ecologists, population demographers, government agencies and business organizations are frequently interested in population-level questions:

- How long do individuals in a population live?
- What ecological and historical factors affect longevity?
- Do certain identifiable groups in a population have distinctive mortality rates and patterns?

Data appropriate for analysis can be collected in two general ways. A *vertical* approach follows a cohort (group of individuals) born at the same time. Using this method, for instance, one could record the names of babies born within a single time period in a city or other designated area and keep track of them until they die. If the data were organized into a life table, they would yield information about age-specific mortality, survivorship and life expectancy. Long-lived organisms clearly pose certain problems when using the vertical approach. A second, more generally utilized method, the *horizontal* approach, analyzes data on all ages within a given population at one time. This method has the distinctive advantage of allowing one to collect a large amount of data in a relatively short time and is the method discussed in this article.

Methods

Any plant or animal species for which a record of birth and death has been kept can be used for a population study. The species *Homo sapiens* is particularly amenable to examination. It is an abundant, large mammal that has the curious habit of burying dead individuals at particular sites called cemeteries. Furthermore, information on gender and dates of birth and death are often recorded at the site on tombstones. Interesting historical information can be gleaned from group burials: a disastrous fire that killed a number of firefighters, a massacre during the early settlement of a town, or war-related deaths. Any community in the United States has at least one prominent cemetery, and many more are available in the surrounding countryside. We propose that these sites be used to teach students how to collect, analyze and present data; as well as to research the history of their community.

A simple, but interesting study can be conducted by a single student or cooperatively by a single class or group of classes. Before data collection begins, contact the curator of the cemetery regarding the proposed study. We have found curators to be helpful with historical information regarding the cemetery and the region. Entry into older cemeteries is often restricted; contact the local city hall to inquire how to access the cemetery. Remind students to be respectful when collecting cemetery data.

Cemeteries are usually well organized, and it is easy to arrange students in rows to prevent overlap. As students move through the cemetery, they should record gender and dates of birth and death. If a name has historical importance, it should be recorded. Clipboards are helpful, but notebooks are sufficient. Because in older cemeteries it is often difficult to read the information on tombstones, a crayon and paper can be used to make tracings. If the entire cemetery is not surveyed, it is important to randomly collect data from all parts and estimate the percentage of the cemetery surveyed.

Results & Discussion

Data can be organized by age groups, gender, cause of death (if stated), etc. and presented using population pyramids, histograms and/or line graphs. Graphs can be constructed using a computer graphics program or by having students draw them.

Math can be integrated in various ways. Means, ranges, medians, etc. can be generated using a calculator or recorded on a spread sheet if computers are available. Data can be analyzed for significant difference using a statistics program or simply using a calculator.

In 1989, we participated in Southern Illinois University at Edwardsville's Black Hills Field Trip. After learning about the history of Deadwood, we became interested in changes of human life expectancy throughout Deadwood's history. Data were collected from more than 1100 tombstones in two cemeteries. A histogram was constructed to show the changes in average length of life over time (Figure 1). Notice the increase in age at death of Deadwood citizens from 1860 to 1960. Students could interpret the graph, hypothesize explanations

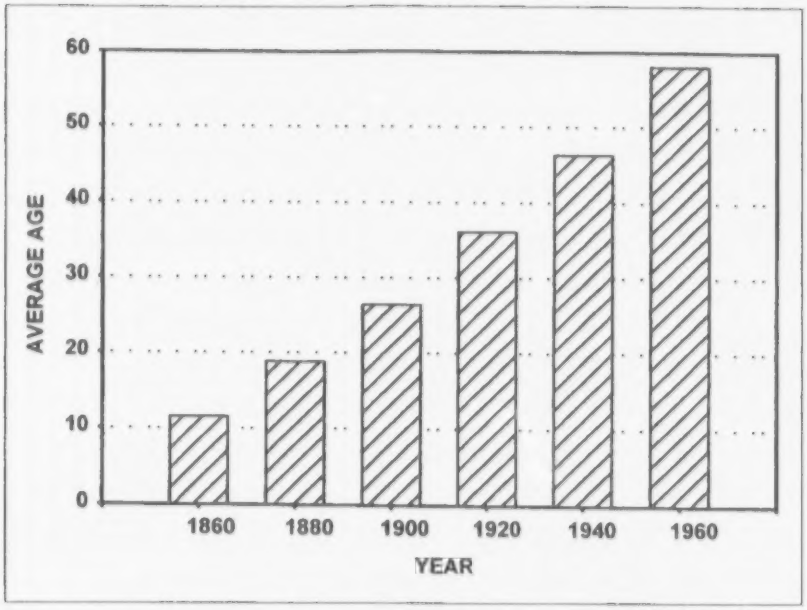

Figure 1. The average age of citizens in Deadwood, South Dakota by 20 year periods, 1860–1960.

for the increase in age at death, and research their hypotheses in their social studies class or by using references in the library.

Population pyramids are useful when observing age structure of a population at a specific time. Figure 2 displays the population age and gender structure for Deadwood in 1860, which can be compared to that of 1920 (Figure 3). Notice the shift from a young to an older population. Students can also compare these data to Figure 1. Do Figures 2 and 3 reinforce the information given in Figure 1? What are the advantages of population pyramids compared to histograms?

Data from a particular location can be compared to that from another location. The State Center for Health Statistics is a good source for county and state statistics. This office can answer many questions that may be of interest to a social studies class:

- How are statistics collected and filed?
- What kind of information is part of the public record and available to all citizens?
- How do government agencies work?
- Why is it important to have and maintain such government agencies?

A class tour or a guest speaker from the state records department may be of interest. State and national vital statistics documents are found in most university and large public libraries. In Figure 4, male/female mortality ratios in Deadwood are compared to those of the United States (Dublin et al. 1949). Notice the decrease in the ratio. Students often have difficulty understanding ratios. What exactly does a male/female mortality ratio indicate? What would cause the ratio to increase or decrease?

A comparison of male and female life span is often of interest. In Deadwood, SD, males outlived females until the 1920s (Figure 5). Students could compare Figure 5 to the male/female mortality ratios in Figure 4. After developing hypotheses, students could research the changes in female length of life. Using library resources, we found that there were important refinements in Cesarean sections during this time period (Guttmacher 1973). The improvements in maternal care greatly increased female life span. Another interesting fact was that the occurrence of the Korean War, 1950–1953, may account for the slight decrease in male life span and increase in female life span. While men were away at war, fewer women were pregnant and as a result female mortality due to childbirth decreased. Topics such as these may interest students and could be researched in more detail.

In 1995, two students from East Alton-Wood River High School in Wood River, Illinois conducted a similar study. They collected data from local cemeteries and researched local history using newspaper archives, the school library, public library and interviews with local people regarding community history.

Data from 1,992 tombstones dating 1835 to 1994 were collected from East Alton, Wood River, and Bethalto. With the permission of the City of Wood

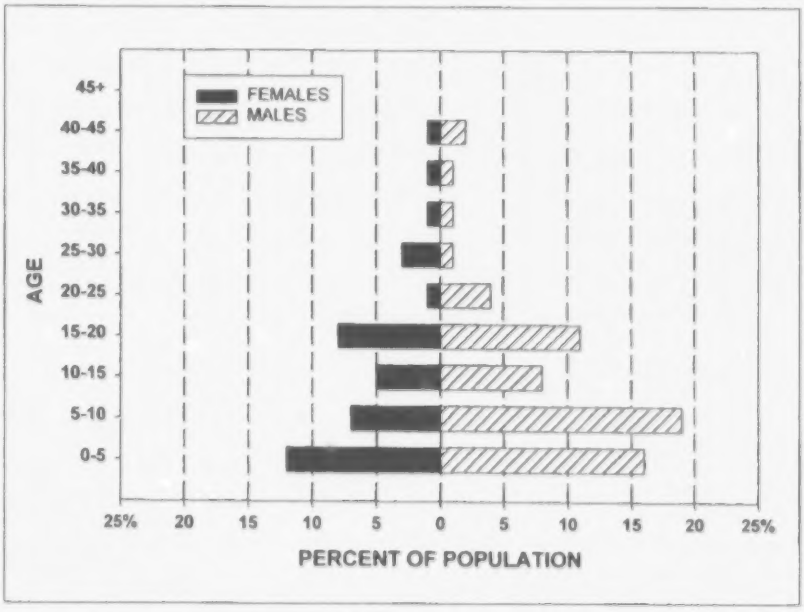

Figure 2. The population age structure of females and males of Deadwood, South Dakota in 1860.

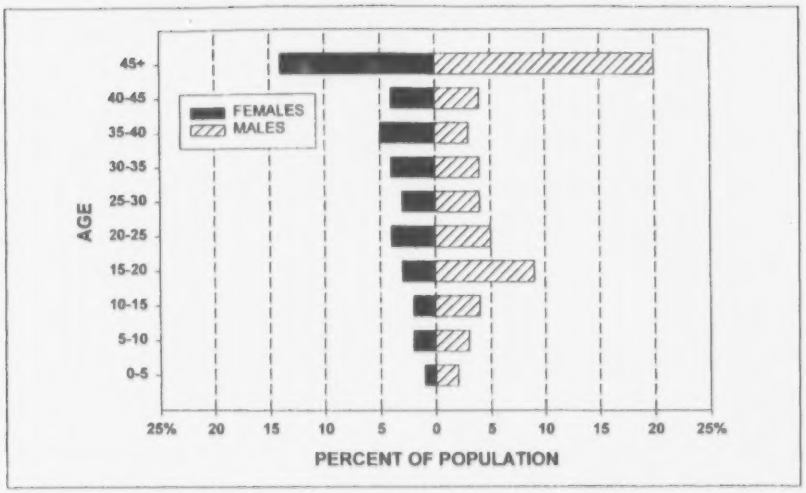

Figure 3. The population age structure of females and males of Deadwood, South Dakota in 1920.

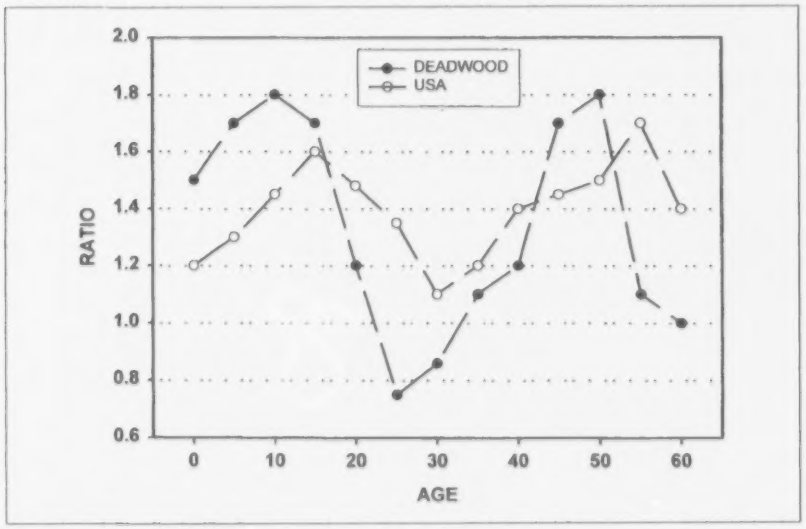

Figure 4. The male/female mortality ratio of Deadwood, South Dakota (1920) and USA (1939–1941).

River, data were collected from Vaughn Hill cemetery which was established in 1800. Due to vandalism and erosion, many tombstones were damaged; therefore, only 75% of the cemetery was surveyed.

Figure 6 illustrates the average length of life of males and females for the Wood River area from 1835 to 1994. Data for the 1800s consisted of relatively small sample sizes from private cemeteries. Notice the similar increase in female longevity after 1915 as seen in Deadwood. Students could identify differences in community structure trends from 1835 to 1994. What do these data say about population trends in the two cities? Comparisons of data from different parts of the country are often interesting. Students could compare mortality data from different parts of the United States in social studies class and identify trends across the country.

This project also lends itself to sharing information beyond the classroom. Data that were collected and analyzed could be used to write a scientific paper to fulfill a technical writing requirement. Summaries of the project could be published in school and local newspapers. An oral presentation or a poster could be presented at the school's Open House or a community function.

This activity was designed as an inexpensive way to teach students how to collect, analyze and present data. It can easily be used as an interdisciplinary study including biology, math, social studies and English. Or, it could be completed by a class within a single discipline. The activity can be adapted in many ways: a brief summary of changes in average life span of a community, an in-depth look at the local mortality history, or a detailed analysis of local life span related to local history and compared to national mortality.

In conclusion, population data are not simply an array of figures organized on a table or graph. They tell the story of human effort to prolong the existence to which they instinctively cling. Life span is affected by biological factors, including genetics, gender and disease; environmental factors, such as weather; wars; and advances in technology and medicine. Human population studies can provide a simple, inexpensive and interesting research project for students.

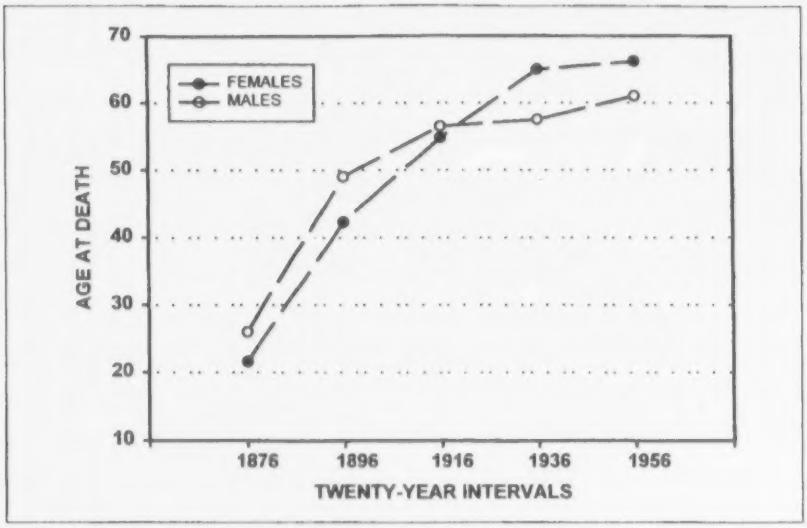

Figure 5. The average life span of males and females in Deadwood, South Dakota, 1876–1975.

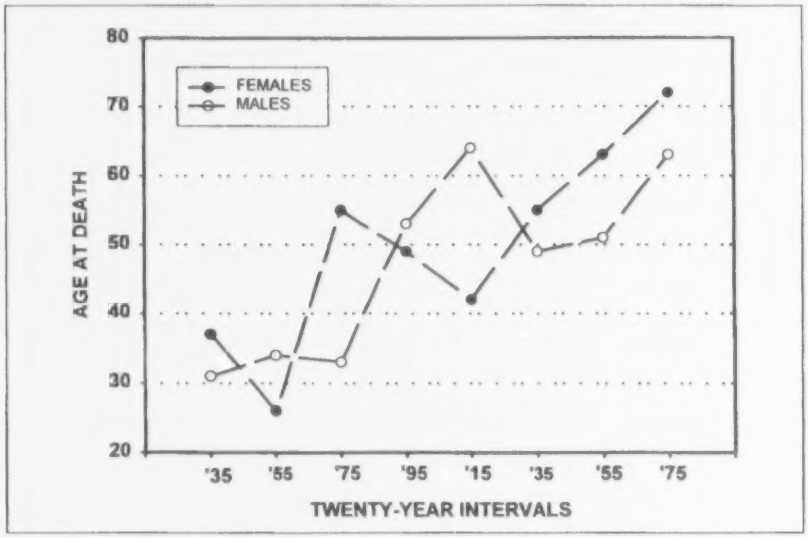

Figure 6. The average life span of males and females in Wood River, Illinois, 1835–1975.

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Pond in a Jar

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I have found microcosm laboratory exercises to be quite successful with my students, especially in introductory biology and environmental biology courses. Initially described by Taub (1969) as a means for observing and manipulating single natural phenomena, simulations allow students to investigate important principles of ecology such as succession, predator-prey relationships, population dynamics, and the effects of manipulating various environmental factors, in a laboratory situation. Investigations with microcosms are also becoming an important area of sophisticated research in modern ecology (Odum & Deyers 1992). There is a growing body of research literature using microcosm techniques (e.g. Larsen et al. 1986 and Taylor et al. 1990). Ecologists are using very elaborate microcosms to simulate natural environments (Adey 1992). The studies are limited in that whole ecosystem responses cannot be observed, but still provide valuable insights into ecosystem processes. These techniques are also being used in the classroom (Corner 1992; Jones 1992; and Murphy et al. 1992) to give students hands-on experiences.

I have tried a number of different types of microcosms, such as hay infusions and bottle biology projects. One of the most successful activities is what I call a "pond in a jar." I modified this activity from Taylor and Kaufman (unpublished). It is an easy way to set up a self-sustaining ecosystem and it is very versatile. The simple and inexpensive exercise works for students at almost any level, elementary school to graduate school. It can be used as a simple observation activity or to perform a variety of experiments. Field trips can be combined with a long-term indoor activity or the "pond in a jar" can be a substitute when field trips are impractical.

Materials

The following materials are needed to assemble the microcosm.

- Glass or plastic wide-mouthed quart jar (Jars with plastic lids work best as they do not rust; bigger jars are okay, but smaller sizes do not work very well).
- Plankton nets (I use commercial plankton nets, but handmade nets of stocking or other material could substitute).
- Water samplers (I use a Van Dorn Sampler, but homemade equipment could substitute).
- Small hand trowel.
- Bottom sampler (I use a Peterson Dredge, but a shovel will suffice if that is all that is available).
- A light setup or a well-lighted window.

Procedure

I take my classes on a field trip to a local park that has a pond in it; however, if field trips are impossible, the instructor can do the collecting and bring the materials to campus. The students then assemble their microcosms in the classroom. I have my students bring their own jar and I supply the sampling equipment. I teach the students how to use the various samplers. Students then prepare their "pond in a jar."

Preparing the Microcosm

- Take a sample of bottom mud with the dredge.
- Place an inch or two of mud in the bottom of the jar (There should be some benthic organisms in this mud).
- Take plankton samples and add them to the jar (This provides a nice group of microcrustaceans—cladocerans, copepods, etc.—and algae).
- Place some sticks, rooted aquatic plants, filamentous algae, duckweed, etc. into the jar.
- Fill the jar up with pond water and cap it.
- Return the jars to the lab for further observation and experimentation.

It is not a good idea to place larger organisms, such as fish and insects, in the jar. I have had students ignore this and place small minnows in their jars. The minnows have survived an entire semester. The jar should be kept closed. The ecosystem will remain functioning for a very long period, given adequate light. I have a light setup and keep the jars under fluorescent lights. The lights are on a timer and stay on 14 hours per day. These ecosystems can be stored in the dark and brought out at a later time. They rejuvenate when placed in light.

Investigations

Students may pursue a variety of investigations using their microcosms. The following is a list of some of the possibilities.

- Measuring pH and other physico-chemical parameters over time.
- Successional studies done by keeping records on the organisms they find in their jars over time. My students have found leeches, hydra, amphipods, copepods, cladocerans, ostracods, midge larvae, various types of algae, snails, etc.
- Controlled investigations using paired jars (experimental and control) to determine the effects of:
  1. Different lighting conditions
  2. Fertilization with inorganic nutrients
  3. Organic enrichment, such as adding sucrose
  4. Inorganic and organic pollution.

Conclusion

The "pond in a jar" is a simple and inexpensive way to bring ecology into the classroom. It is very versatile and can be adapted for life science courses at all levels.

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Measuring the Species Diversity of Freshwater Plankton Communities

A Field & Classroom Exercise

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A central theme in biology is the vast diversity of life. In recent years, it has become apparent that the decline of the Earth's biodiversity will be one of the important problems facing students in coming decades. In this paper, we describe a simple exercise that gives students both a field and classroom experience in measuring biodiversity. We have developed this exercise so that students can:

1. Learn how to make and use a simple plankton sampling device.
2. Gain experience in counting microscopic organisms.
3. Use a species diversity index to compare plankton communities.
4. Develop and test hypotheses concerning plankton diversity in different aquatic ecosystems.
5. Become familiar with an often overlooked but diverse and dynamic group of organisms.

At the present time there are about 1.4 million species of microorganisms, animals and plants that have been studied and given scientific names. Of these organisms, the great majority are insects (approximately 750,000 species) and flowering plants (approximately 220,000 species). These numbers, however, may represent only a small fraction of the actual biodiversity on our planet, various estimates place the total numbers anywhere from 10 million to more than 100 million different types of organisms (Ehrlich & Wilson 1991).

In the past few years, biologists have become very concerned over an accelerated rate of species extinction and reduction of biological diversity. This concern has been particularly focused on the destruction of habitat in the tropical rain forests. Estimates suggest that 4000 species may become extinct per year due to deforestation in the tropics alone (Wilson 1989). On a global scale, it appears that we are losing 100,000 species annually due to human expansion and resultant loss of habitats. According to some estimates, the current rate of habitat loss will result in the extinction of 25% or more of all species on Earth in the next 50 years (Ehrlich & Wilson 1991). As illustrated by the recent "Earth Summit" in Rio de Janeiro, solutions to the biodiversity problem are not readily forthcoming in the face of enormous social, political and economic pressures.

Most students are acquainted with the concept of biological diversity but often only have a vague definition that they can apply to it. Furthermore, they are under the impression that biological diversity is important only in tropical jungles or other exotic locales. In reality, the planktonic communities existing in local, temperate ponds or lakes are quite complex and are readily available for analysis at most times of the year.

Simpson's Index of Diversity

In the study of an ecological community, one of the key questions deals with the type and abundance of species. In effect, the researcher asks, "How rich and varied is the community?" To help answer this question and to qualify species diversity, a number of species diversity indices have been developed (Magurran 1988). We have chosen an easily computed form of Simpson's index to calculate plankton diversity (Krebs 1985). This index is derived from probability theory and basically predicts the chance of simultaneously picking two members of the same species when sampling a large community. Little weight is given to rare species and greater weight is given to the more common species. The numerical value for this index ranges from zero (low diversity) to a maximum of 1-1/S, where S is the number of species in the community.

$$D = 1 - [(p_1)^2 + (p_2)^2 + (p_3)^2 + \ldots . (p_n)^2]$$

or

$$D = 1 - \Sigma(p_i)^2$$

where D = Simpson's Index of Diversity

P_i = the proportion of species i in the community (i.e. # of individuals of species i ÷ total # of individuals in community)

This index is most meaningful when it is compared to the maximum theoretical diversity (1 − 1/S) for the community.

The "Ajax" Plankton Sampler

Several types of plankton sampling devices are commercially available but also are quite expensive. We devised a simple and inexpensive sampler that is effective in collecting plankton at controlled depths (Figure 1). This sampler was made from a plastic laundry detergent bottle (Ajax® liquid laundry detergent, 64 fl. oz.) that was taped to a pole (in our case, an aluminum pole with 0.5 m markings). The bottle was capped with a rubber tank ball that was held in place with stretched latex tubing (7.5 mm, O.D.). The elasticity of the latex tubing created a tight seal. A gentle tug on a nylon cord, attached to the tubing about the tank ball, allowed the bottle to fill at a specific depth. Releasing the cord resealed the Ajax® sampler.

The sample was then poured through a standard, upright plankton net to concentrate the organisms (a fixative may be added if the sample is to be stored for any period of time). Measurement of the volume of the sampling bottle (this changes slightly with depth) and the final volume of

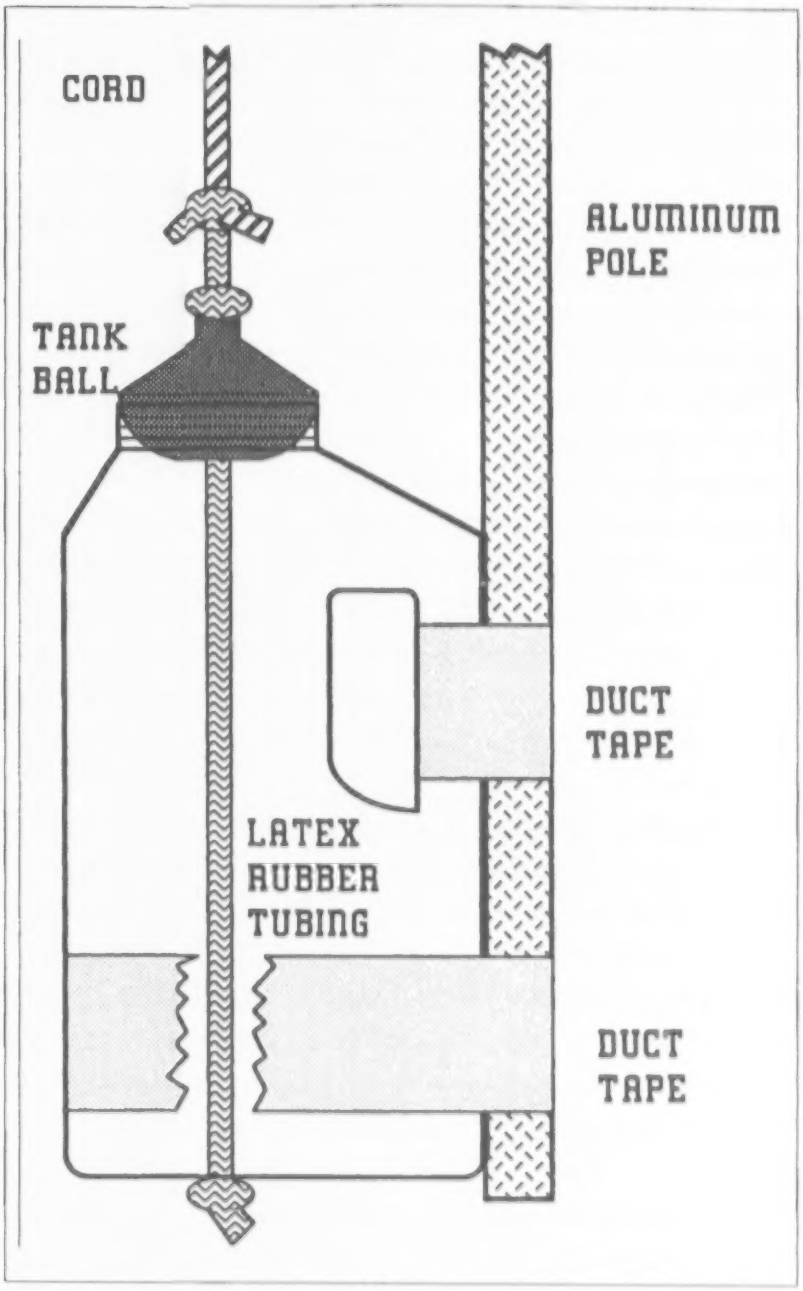

Figure 1. The Ajax® plankton sampler. A liquid laundry detergent bottle was taped to an aluminum pole and the bottle was sealed with a tank ball. The elasticity of the latex rubber tubing created a tight seal. At a specified depth, the cord was pulled to allow filling and then released to reseal the bottle.

the concentrated sample allow the student to calculate the number of organisms per liter of water.

The Counting Cell

In plankton studies, the Sedgwick-Rafter counting cell is typically used to determine the number of organisms in a concentrated sample. This counting cell is commercially available (see Lind 1979 for a description), however, we have constructed an inexpensive alternative and have found it works well (Figure 2). We constructed this counting cell from a standard glass microscope slide and a plastic microscope slide of the same size. Using a razor blade, a rectangular opening (50 × 20 mm) was cut in the plastic slide; the remaining plastic border was bonded to the glass slide with a flexible, waterproof glue. A portion of the water sample was then placed in the "well" and an intact plastic slide used as a cover slip. The volume held by the well depends on the type and amount of glue used. Uniform copies of the counting cell can be produced by carefully dispensing the glue and by placing weights on the counting cell as the glue sets; excess glue is easily trimmed with a razor blade after curing. Our counting slides comfortably hold 0.6 ml.

Plankton Identification

Identification of planktonic organisms to the genus or even species level is not really necessary to compute a species diversity index. As long as students can recognize one species as distinct from another, scientific names are not essential. However, we feel that identifying organisms to at least the genus level enhances the students' appreciation of this community of organisms. Some useful references that can aid students in plankton identification are listed in the References section. In the laboratory, we provide the students with photocopies of key pages from Needham & Needham (1962).

Experimental Procedure

Students can use the following protocol for studying plankton diversity:

1. Collect water samples, at specified depths, using the Ajax® sampler. Usually five or more samples should be collected at a specific site, combined and concentrated with a plankton net. If desired, the concentrated sample can be preserved by adding formaldehyde or alcohol (final concentration = 4% formaldehyde or 10% ethanol).
2. In the laboratory, transfer 0.6 ml (this volume may differ, depending on construction technique) to a counting cell and cover with a plastic slide. The sample vial must be gently stirred before taking this sample. At 100X, scan the entire slide and identify all observed plankton species (microscopes with mechanical stages are desirable for scanning the counting cell). Use the "Organism Guide" to identify each species.

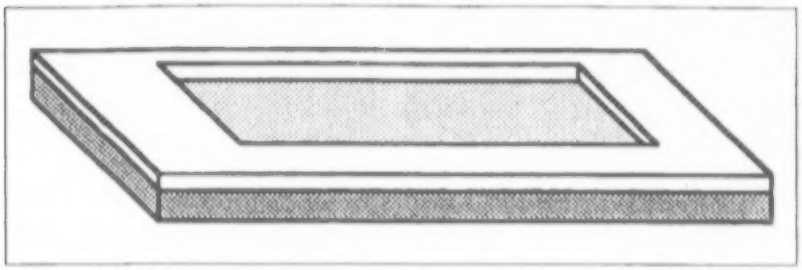

Figure 2. Counting cell. A plastic microscope slide with a rectangular cut-out was glued on top of a standard glass microscope slide. A second, intact, plastic slide was used as a cover slip after a plankton sample was placed in the well.

Table 1. Plankton counts from a spring pond sampled at a depth of one meter with the Ajax® sampler.

Sample Description: 60 m² Spring Pond

Species	*Number*	*Proportion* (p_i)	(p_i)²
Algae			
Synedra	500	.7645	.5845
Navicula	55	.0841	.0071
Gyrosigma	27	.0413	.0017
Frustulia	33	.0505	.0025
Rotifera			
Philodina	18	.0275	.0008
Crustacea			
Chydorus sphaericus	21	.0321	.0010
Total Individuals = 654		$\Sigma(p_i)^2$ = .5976	
Number of Species = 6			
Max Diversity (1-1/6) = .83			
Simpson's Index = .40 ($D = 1 - \Sigma(p_i)^2$)		Simpson's Index as % of max. = 48.3%	

This identification and counting procedure works well if students work in pairs; one student scans the slide and identifies species while the other student records data.

3. Repeat this procedure for each field sample.
4. For each field location determine the following from your sample counts:

   Number of species
   Total number of individuals
   Density of organisms (see below)
   Simpson's Index of Diversity

   $$\text{density of organisms (\#/liter)} = \frac{\text{total \# individuals counted}}{0.0006 \text{ liters}} \times \frac{\text{volume in sample vial (l)}}{\text{volume filtered (l)}}$$

5. Students should be made aware of environmental variables that may affect plankton distribution and density. Therefore physical and chemical data, such as water temperature, light penetration (turbidity), wind speed and direction, pH, and substrate characteristics, should be collected at each sampling site.

The laboratory handout used by students for this exercise is available from the authors on request.

An Example

The Ajax® sampler was used to collect plankton from three lentic ecosystems: a small spring pond (approximately 60 m²), a campus research pond (approximately 0.4 hectare), and a lake of several hundred hectares located on a wildlife refuge. At each location, five Ajax® plankton samples were collected at a depth of one meter, and the samples were combined and concentrated into a vial by filtering through a plankton net. These samples were preserved as 4% formaldehyde solutions, although 10% ethanol would also suffice. In the laboratory, three subsamples were analyzed from each site, and the number of species and number of organisms per species were determined.

Tables 1–3 show the plankton community data collected from each location and the computations needed to determine Simpson's Index of Diversity. (These tables could be used as models to construct student worksheets.) Comparison of Simpson's diversity indices shows that two of the lentic ecosystems have nearly identical diversities—values representing 81% of maximum theoretical diversity. This is due to the similar number of species at both locations and the even distribution of individuals among these species. The small spring pond has a relatively low diversity (48% of maximum) since the community is dominated by one species.

An alternative expression of the species diversity formula (Cox 1980) has been included with an example data set (Table 4). This formula may be useful if the students do not have a solid math background.

A Test of the Ajax® Sampler

We were concerned about whether our collecting device would accurately estimate the number of species present and the number of individuals per species. Therefore we compared the Ajax® sampler with a commercially available van Dorn sampler (see Lind 1979 for a description). This device is designed to collect water chemistry samples but also is frequently used to collect plankton samples at specified depths. The number of plankton species, including rare organisms, collected from a small pond at two depths were virtually the same (Table 5). The Ajax® sampler, however, gave lower estimates of densities than the van Dorn sampler. A comparison of Simpson's Index of Diversity produced by the two samplers showed no significant statistical differences (tested via ANOVA). Therefore we feel confident that the Ajax® sampler will give reliable and reproducible estimates of plankton species diversity.

Table 2. Plankton counts from a small research pond sampled at a depth of one meter with the Ajax® sampler.

Sample Description: Small Pond (0.4 hectare)

Species	*Number*	*Proportion* (p_i)	(p_i)^2
Algae			
Synedra	27	.1007	.0101
Fragilaria	123	.4590	.2106
Gomphonema	13	.0485	.0024
Navicula	3	.0112	.0001
Oedogonium	27	.1007	.0101
Ulothrix	3	.0112	.0001
Protozoa			
Strombidium	3	.0112	.0001
Rotifera			
Asplanchna	13	.0485	.0024
Pleurotrocha	5	.0187	.0003
Keratella cochlearis	5	.0187	.0003
Filinia	3	.0112	.0001
Crustacea			
Chydorus sphaericus	19	.0709	.0050
Bosmina longirostris	3	.0112	.0001
Daphnia pulex	5	.0187	.0003
Nauplius larvae	16	.0597	.0036

Total Individuals = 268 — $\Sigma(p_i)^2$ = .2455
Number of Species = 15
Max Diversity (1-1/15) = .93
Simpson's Index = .75 — Simpson's Index as % of max. = 80.8%
($D = 1 - \Sigma(p_i)^2$)

Table 3. Plankton counts from a wildlife refuge lake sampled at a depth of one meter with the Ajax® sampler.

Sample Description: Wildlife Refuge Lake (> 100 hectares)

Species	*Number*	*Proportion* (p_i)	(p_i)^2
Algae			
Synedra	70	.1449	.0210
Asterionella	38	.0787	.0062
Navicula	3	.0062	0
Cymbella	9	.0186	.0003
Pediastrum	41	.0849	.0072
Rotifera			
Asplanchna	9	.0186	.0003
Keratella cochlearis	14	.0290	.0008
Brachionus calyciflorus	14	.0290	.0008
Polyarthra	11	.0228	.0005
Crustacea			
Chydorus sphaericus	14	.0290	.0008
Bosmina longirostris	41	.0849	.0072
Daphnia pulex	3	.0062	0
Nauplius larvae	216	.4472	.2000

Total Individuals = 483 — $\Sigma(p_i)$ .2451
Number of Species = 13
Max Diversity (1-1/13) = .92
Simpson's Index = .75 — Simpson's Index as % of max. = 81.8%
($D = 1 - \Sigma(p_i)^2$)

Table 4. Alternative form of Simpson's Index of Diversity.

$$D = \frac{N(N - 1)}{\Sigma n(n - 1)}$$

Where: D = diversity index
N = total number of individuals of all species
n = number of individual of a species

Example (Data from Table 1)

Species	*Number (n)*	*n(n − 1)*
Algae		
Synedra	500	249500
Navicula	55	2970
Gyrosigma	27	702
Frustulia	33	1056
Rotifera		
Philodena	18	306
Crustacea		
Chydorus sphaericus	21	420
	N = 654	$\Sigma n(n - 1)$ = 254954

$N(N - 1) = 427062$

$$D = \frac{427062}{254954} = 1.67$$

Student Projects

The general experimental procedure presented in this paper can be used by students to test hypotheses concerning the species diversity of aquatic ecosystems. Some factors that affect plankton diversity and can be incorporated into student studies include vertical distribution, temporal distribution, light penetration (turbidity), nutrient enrichment, other forms of pollution, water temperature and pH. This exercise is especially well-suited as a scientific investigation and writing project. We have used this exercise in an introductory-level college, biology laboratory with successful results but feel that it could be incorporated into an advanced high school biology or ecology course. However, at least four hours of laboratory time are needed for most student teams to count and identify organisms in the counting cells. The presence of the instructor is essential during, at least, the initial part of the counting. This exercise may be of greatest value as a longer term project covering, perhaps, six to eight hours of class time (including both field and laboratory portions).

Table 5. A comparison of values obtained with the Ajax® sampler and a commercially available van Dorn sampler. Values are presented as the mean and range of five samples. The Ajax® sampler reliably measures the numbers of species, the number of rare species and Simpson's Index of Diversity. This device, however, underestimates the total density of organisms.

Type of Sampler	*Number of Species*	*Number of Rare Species*	*Density (organisms/l)*	*Simpson's Index*
Ajax®, surface	10.8 (10–12)	8.0 (7–9)	2091 (1166–3078)	.52 (.47–.58)
van Dorn, surface	10.2 (8–12)	7.4 (6–9)	4615 (2747–6324)	.53 (.41–.57)
Ajax®, 1 m	12.4 (9–15)	6.8 (4–9)	4824 (3090–6573)	.50 (.49–.54)
van Dorn, 1 m	9.6 (7–14)	5.8 (2–10)	6480 (4569–9197)	.51 (.47–.55)

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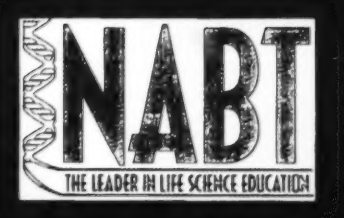

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